

# IMMUNOCAPILLARYMIGRATION

and two other rapid methods for  
immunochemical quantitation

by

CRISTINA GLAD

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THE LUND INSTITUTE OF TECHNOLOGY

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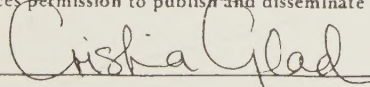


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| Abstract<br><p>A new rapid method for immunochemical quantitation - immunocapillarymigration - is presented. It is based upon the capillarymigration of an antigen-containing sample in a porous material to which antibodies have been attached. Through interaction with the antibodies the antigen will be specifically retarded in its migration related to all other constituents in the sample. This retardation will increase with decreasing antigen concentrations in the sample. Antigen-covered areas are thus obtained and these are exposed by labelled antibodies. The heights of these areas increase with increasing concentration of antigen in the sample.</p> <p>The choice of porous material, methods for attachment of antibodies, different exposure techniques and the mechanism of immunocapillarymigration have been investigated. It was found that by the use of antibodies labelled with horse-radish peroxidase to expose the antigen-covered area, C-reactive protein (CRP) could be determined down to 0.15 mg/l (1.25 nmol/l) with a coefficient of variation of 7%. A comparison with electroimmunoassay gave a correlation coefficient of 0.95. When chromatography paper was used as the porous support proteins could be quantified within 15 min. The relation between the amount of antibodies coupled to the porous material and the concentration of antigen that could be analyzed was studied. These two parameters were proportional to each other.</p> <p>A modification of electroimmunoassay - high voltage electroimmunoassay - is presented. A combination of low ionic strength and a high electric field strength allowed very rapid determinations of antigens. The method was used to quantify several plasma proteins and results were obtained within 9 min for albumin and within 25 min for IgG.</p> <p>A quantitative version of the Laurell type of crossed immunoelectrophoresis is also presented. An internal standard, reduced and carboxymethylated ovalbumin, was used. The method allows rapid quantitative determinations of different molecular forms of a protein.</p> |                         |                                        |       |
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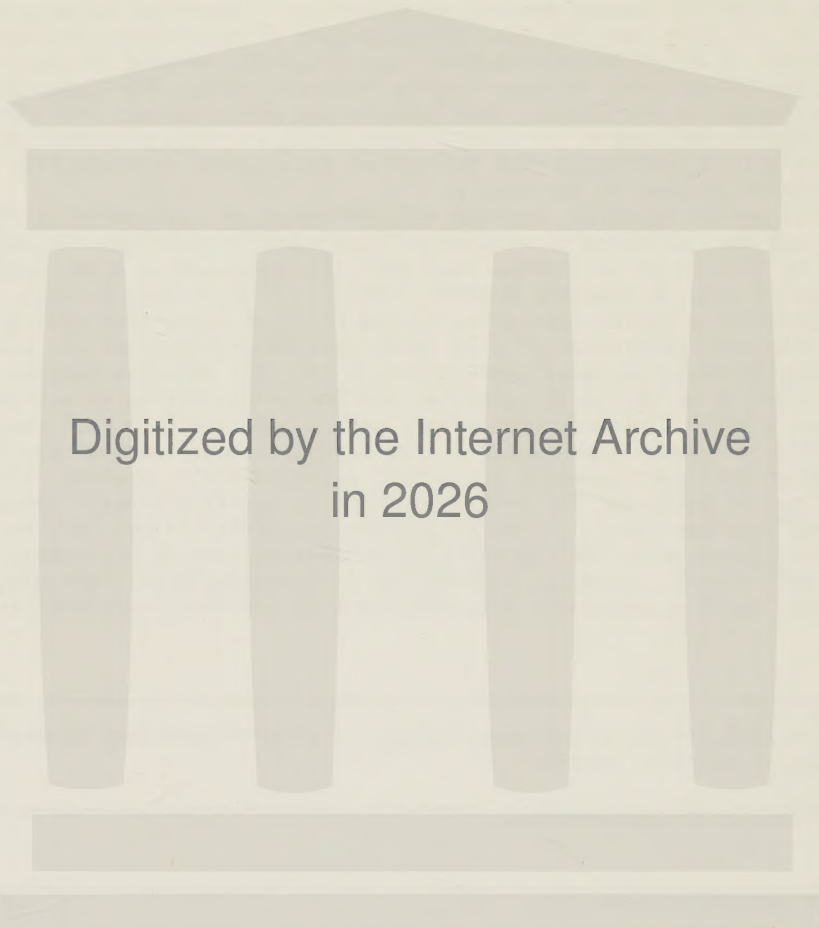
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This dissertation is based on the following papers, which are referred to by Roman numerals in the text.

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Biochem. Soc. Trans. 5 (1977) 712-714
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Immunocapillarymigration - A new method for immunochemical quantitation.  
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- IV. Glad, C.  
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High voltage electroimmunoassay - An ultrarapid method for immunochemical quantitation.  
Scand. J. Immunol. Suppl. 9 (1981) in press
- VII. Grubb, A.O., Glad, C., Grimsberg, V. and Thysell, H.  
Quantitative crossed immunoelectrophoresis - A convenient procedure for the simultaneous quantitation of various molecular forms of an antigen.  
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## I N T R O D U C T I O N

Antigen-antibody interactions form the base for a variety of analytical methods. The specificity and sensitivity of these methods have led to their use in many different diciplines outside the field of immunology. The work by Heidelberger and Kendall (1935) on the principle of quantitative estimation of proteins by precipitation has initiated the development of such methods.

The antigen-antibody interaction occurs in two steps. The first and specific step, the primary reaction, is the binding of antigen to antibody. The second step can be of different kinds, e.g. precipitin formation, agglutination of cells or hemolysis. Both the antigen-antibody reaction and the secondary reactions have been used for analytical purposes. The precipitin formation is used in e.g. quantitative gel immunodiffusion, immunoelectrophoretic techniques, and nephelometry.

Immunodiffusion techniques are of two types. In single immunodiffusion the antigen diffuses in an antibody-containing gel either linearly in a tube (Oudin, 1946) or radially from a well into the gel on a plate (Mancini et al., 1965; Fahey and McKelvey, 1965). In double immunodiffusion both antigen and antibody are diffusing in the gel (Ouchterlony, 1948; Wieme and Veys, 1970).

In the original immunoelectrophoretic technique (Grabar and Williams, 1953; Scheidegger, 1955) the antigens were first separated by electrophoresis and then identified by double immunodiffusion. This technique was qualitative only. In crossed

immuno-electrophoresis (Laurell, 1965) the immunodiffusion step was replaced by electrophoresis of the separated antigens into an antibody-containing gel. This semi-quantitative technique was later modified to a quantitative procedure (Clarke and Freeman, 1967; 1968). The quantitative properties of crossed immuno-electrophoresis are also utilized in electroimmunoassay (Laurell, 1966) where antigen is electrophoretically moved into an antibody-containing gel.

In nephelometry, antigens are quantified by light scattering measurements of the precipitin reaction (Schultze and Schwick, 1959). This technique has been developed after several lines. Automatic versions either based upon steady state (Ritchie et al., 1973) or kinetic (Sternberg, 1977) measurements of the reaction are available. By the use of monochromatic light provided by a laser, the sensitivity of the technique has been improved considerably (Deaton et al., 1976).

The specific primary interaction between antigen and antibody is used in very sensitive quantitative methods as radio immunoassay (Yalow and Berson, 1959) and enzyme immunoassay (Engvall and Perlmann, 1971). These methods are based upon the competitive binding of labelled and non-labelled antigens to limiting concentrations of antibodies. Bound and free antigen can be separated by e.g. precipitation (Farr, 1958), adsorption on charcoal, immuno-adsorbents (Catt and Tregear, 1967), or the use of polymer two-phase systems (Mattiasson, 1980).

This dissertation presents a new immunochemical method in which antigens is quantitated by the primary interaction between antigen and antibody obtained during capillary migration of antigen in an immunosorbent (I-V). A modification of electroimmunoassay allowing rapid quantitations of antigens (VI) and a modification of crossed immuno-electrophoresis according to Laurell for quantitative measurements (VII) are also included in the dissertation.

## IMMUNOCAPILLARYMIGRATION

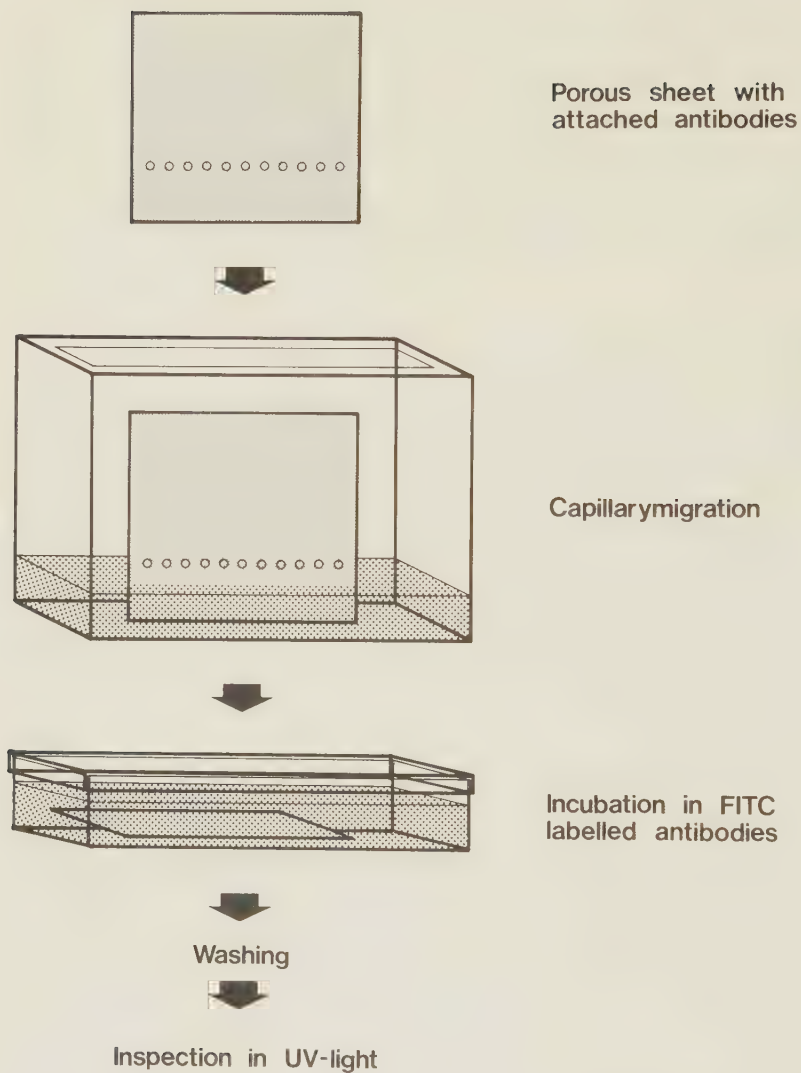
### INTRODUCTION

Immunocapillarymigration is a method for immunochemical quantitation that utilizes the capillary force of an immunosorbent to bring about the necessary antigen-antibody interaction. The immunosorbent, which is composed of a porous sheet with attached antibodies, can be used in two different ways. Several samples may be analyzed on the sheet simultaneously or, alternatively, each sheet is cut into strips and one strip is used for each sample (Fig. 1 and 2).

The sheet procedure (Fig. 1). Small samples (1-5  $\mu$ l) containing the antigen are applied on the porous immunosorbent sheet, 1-2 cm from the bottom. The sheet is immersed 5 mm into a buffer solution contained in a humidified chamber. The buffer is allowed to migrate by capillary force to a height of 60 mm.

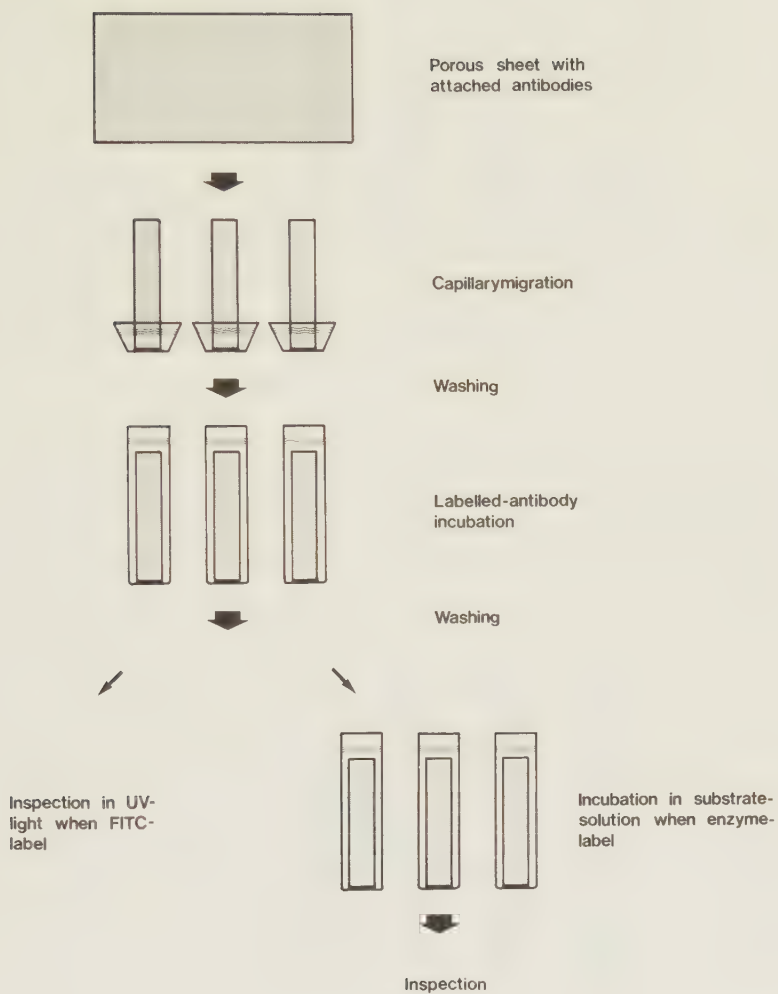
The strip procedure (Fig. 2). The immunosorbent sheet is cut into 5 mm wide strips. Each strip is placed in a cylindrical cup containing 300  $\mu$ l of the sample. The cup is placed in a humidified chamber and the sample solution is allowed to migrate by capillary force to a height of 60 mm.

During migration the antigen in the sample will interact with attached antibodies while all other constituents of the sample will migrate with the solvent front (Fig. 3). The area covered by antigen thus obtained is visualized by incubation in a solution of labelled antibodies (Fig. 1, 2 and 4). Two different kinds of labels have been used, fluorescein-isothiocyanate (FITC) and the enzyme horse-radish peroxidase (E.C.1.11.7). When



*Fig.1: A schematic representation of the immunocapillary migration technique with the sheet procedure.*





*Fig.2: A schematic representation of the immunocapillarymigration technique with the strip procedure.*

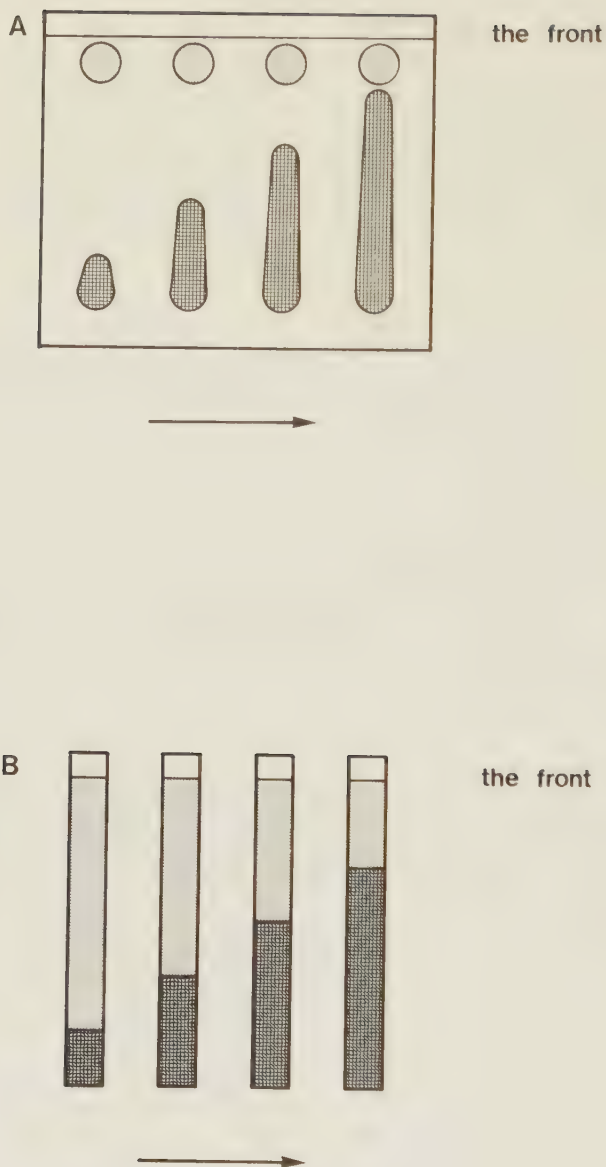
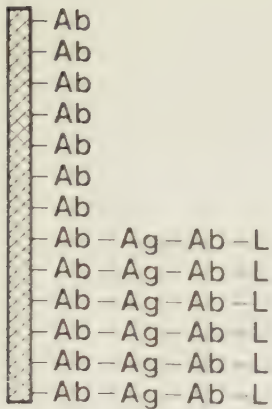


Fig. 3: A schematic drawing of the distribution of antigen and all other constituents in (A) the sheet procedure and (B) the strip procedure after capillary migration. Increasing concentration of antigen in the direction of the arrows.  , all other constituents;  , antigen.



*Fig. 4: A schematic side-view of a strip after the antigen-covered area has been developed.*

Ab = antibody

Ag = antigen

Ab-L = labelled-antibody

FITC-labelled antibodies are used sheets or strips are inspected in UV-light and the limits of the fluorescent areas are marked with a pencil. The use of peroxidase-labelled antibodies requires an additional incubation in a substrate solution, resulting in the development of heavily stained areas where peroxidase-labelled antibodies have bound.

The heights of the fluorescent or stained areas increase with increasing concentrations of antigen in the sample (Fig. 3). The concentration of antigen in the sample can be estimated by measuring the heights of these areas and comparing them with the heights obtained with serially diluted standards.

The immunocapillarymigration method is comprised of the following steps:

1. Choice of porous material.
2. Attachment of antibodies to porous material.
3. Capillarymigration.
4. Detection of the antigen-covered area
5. Quantitative determination.

In the following sections each step will be dealt with in detail.

## CHOICE OF POROUS MATERIAL

A porous supporting material suitable for use in immunocapillary migration should meet the following requirements:

1. Attachment of antibodies should be performed readily.
2. The migration of the sample should be rapid and uniform.
3. There should be no non-specific binding of proteins or of labelled antibodies used to expose the antigen-covered area.
4. The material should be tenacious and easy to handle in both the dry and wet state.

Different porous materials used in paper- and thin-layer chromatography, in filtration or in electrophoresis have been tested. Although there is a great variety of such materials available, there were difficulties in finding one that fulfilled all the above requirements. Most of the materials tested were unsatisfactory because of high non-specific adsorption of fluorescein-labelled antibodies used to expose the antigen-covered area (I,II). A filter material composed of polyvinyl chloride containing silica gel as a filler was chosen. It had good chromatographic properties, antibodies could be attached to it by adsorption, the non-specific adsorption of fluorescein-labelled antibodies was low, and it was tenacious and easy to handle. The migration rate, approximately 60 mm in 30 min, was not regarded as rapid but was acceptable. This supporting material had some draw-backs. First, the consumption of antibodies was rather high; 1 mg IgG was consumed for each strip (II) and the adsorption solution could only be used twice although all IgG molecules were not used up (See: Attachment of antibodies to porous materials). Second, enzyme-labelled antibodies were adsorbed non-specifically to a high degree and, consequently, another material had to be used in the work with this more sensitive detection system (III).

Cellulose acetate sheets, intended for filtration or electrophoresis, did not adsorb enzyme-labelled antibodies non-specifically.



cally (III) in contrast to fluorescein-labelled antibodies (II). Therefore, cellulose acetate was used in the first work with enzyme-labelled antibodies. However cellulose acetate sheets do not fulfil all requirements for a good material. Antibodies can be coupled to the material by, for instance the cyanogen bromide method (Axén et al., 1967) and the migration is uniform, but the material is fragile and the migration rate is slow. When handling the sheets static electricity is a problem. Cellulose acetate is available as mylar-backed cellulose acetate plates. These are more resistant and easy to handle. However, these plates showed a high non-specific adsorption of labelled antibodies and it was also difficult to get a good coupling of antibodies to them.

In the further development of the immunocapillarymigration technique a non-fragile material with a high rate of migration was searched for. Whatman chromatography paper No. 3 fulfilled these two requirements. The migration rate was around 60 mm in 5 min and the paper is tenacious. We knew from earlier work, however, that proteins and in particular labelled antibodies were adsorbed non-specifically to this material (II). This drawback was overcome by incubating the paper in a solution of bovine serum albumin followed by drying before use (V). This chromatography paper is a material that meets the outlined requirements, at least for the applications tried.

#### ATTACHMENT OF ANTIBODIES TO POROUS MATERIALS

Different methods for attaching proteins to solid supports and the evaluation of these methods are topics well covered by the literature on immobilized enzymes and affinity chromatography. The discussion in this section will therefore be limited to the specific problems associated with attachment of antibodies to sheets of porous material and the different methods that have been used.

The solid phase used for affinity chromatography is in the form of beads and the reaction solutions used for activation and for the coupling of ligands can be gently stirred without rupturing the beads. The solid support in immunocapillarymigration is sheets of porous material. Stirring and consequently mixing of the reaction solution during activation and coupling is inefficient even if the sheets can be protected from the stirrer (e.g. by a net covering a magnetic stirring bar). The most efficient mixing is obtained if the container with the reaction solution is gently agitated by hand or by placing it on a rocking table. With this latter technique it is possible to handle several sheets simultaneously. The constant agitation keeps the sheets floating and separated in the reaction solution.

Stirring of the reaction solution is not the only problem associated with attachment of antibodies to porous sheets. Much larger volumes of reaction and washing solutions are needed calculated on a weight basis when compared to beads as the solid support. Even if a reaction vessel is made which fits the shape and size of the sheets, a comparatively large volume will be needed to cover the sheets.

Covalent linkage with CNBr to hydroxyl groups on a solid support as described by Axén et al. (1967) was tested for cellulose and cellulose acetate sheets. It gave a uniform and reproducible coupling of antibodies to the sheets after modification of the method (III). The mixing problem was very obvious when the activation solution had to be kept at pH 10.5 by titration with NaOH during the reaction. The viscosity of the 1-2 mol/l NaOH rendered a uniform distribution of NaOH difficult in the reaction solution. Consequently, pH varied from above 11 at the point of NaOH addition to 8.5 at the most remote point. To avoid titration 2 mol/l  $\text{Na}_2\text{CO}_3$  was instead added to the activation solution (March et al., 1974). Still there was a low degree of coupling, however. A probable reason for this is that the hydrolysis of CNBr in the reaction solution is much faster than the diffusion of CNBr to the surface and interior of the sheets. Therefore, the sheets were first soaked in an unbuffered CNBr

solution in which the pH is too low for a reaction to take place. The sheets were then transferred to a 2 mol/l  $\text{Na}_2\text{CO}_3$  solution to start the reaction. Thus, having the CNBr already at the reaction site, a more efficient activation was obtained and lower concentrations of CNBr were required.

When a beaded solid phase is used the washing steps following activation are performed on a glass filter. Since this was not possible with the sheets the time required for these steps was longer. Through a combination of large volumes of washing solutions and gentle agitation of the reaction vessel it was possible to complete the washing within 10-15 min.

The activated sheets were finally incubated overnight in an antibody solution. Only a small fraction of the antibodies in the solution was coupled to the sheets as estimated by measuring the absorbance at 280 nm before and after incubation. The antibody solution could therefore be used several times.

Crosslinking of proteins with glutardialdehyde in order to make soluble protein complexes has been described by Avrameas (1969). This procedure was tried for attachment of antibodies to cellulose and cellulose acetate. Since crosslinking of antibodies was desired in the porous material rather than in the solution a two-step procedure was used similar to that described above for CNBr-activation. The sheets were soaked in an antibody solution and then blotted with absorbing paper to remove excess solution. The sheets were then incubated in a solution of glutardialdehyde. The degree of crosslinking by this method could be varied by varying the glutardialdehyde concentration keeping the antibody concentration constant. Low concentrations of glutardialdehyde yielded an insufficient crosslinking since antibodies were released from the material on washing. High concentrations, on the other hand, resulted in loss of antibody activity. An optimal result was obtained by combining a 1.5 % (v/v) glutardialdehyde solution with a 2 % (w/v) antibody solution (Pavlu, 1978).

The glutardialdehyde procedure has the advantage that it can be

used with any porous material. However, due to variations in the quality of glutardialdehyde it was necessary to regularly optimize the concentrations to be used in order to get reproducible results.

Coupling of antibodies to chromatography paper oxidized by periodate was found to be a good alternative to coupling with CNBr since it is less toxic (V). Three steps are important when optimizing the procedure. First, impurities in the paper may inhibit oxidation. Therefore, the paper has to be washed carefully before oxidation. Second, the periodate concentration must be adjusted carefully to get a good and reproducible coupling yield without disintegration of the paper sheet (V; Ferrua et al. 1979). Third, the sodium borohydride reduction has to be performed at 4°C. At roomtemperature the hydrolysis of the borohydride is very rapid and hydrogen develops inside the paper sheet and destroys the structure of the paper.

Adsorption of antibodies to the surface of polystyrene tubes (Catt and Tregear, 1967) and to microplates (Voller et al., 1974) is used in many solid phase radio and enzyme immunoassays. Adsorption has the advantage of eliminating the activation step thereby reducing the number of steps that have to be carefully optimized for reproducibility in the attachment of antibodies.

Proteins adsorbed readily to the polyvinyl chloride (PVC) sheets containing silica gel as a filler mentioned in a previous section. It is not clear whether the proteins preferentially adsorb to the PVC or the silica gel of the sheets or perhaps to both materials. It was found in other experiments that proteins adsorb to thin-layer silica gel plates as well as to PVC sheets. The amounts adsorbed to these materials were much less than those adsorbed to the PVC-silica gel sheets. It is difficult, however, to compare the adsorption properties of materials obtained from different manufacturers and produced for different purposes. Stabilizers and plastizicers are present in the PVC product to give the material characteristic properties (Billmeyer, 1971). Hence, the exact composition of PVC obtained from



different manufacturers will vary.

Although adsorption of proteins to plastics is used in both solid phase radio immunoassay (Catt and Tregear, 1967) and enzyme-linked immuosorbent assay (Engvall et al. 1971), few studies of the adsorption process have been performed (Lee and Kim, 1974; Norde and Lyklema, 1978; Cantarero et al., 1980). Therefore, the adsorption of proteins to the PVC-silica gel sheets were studied to answer the following questions: Does the amount of protein adsorbed vary with pH and ionic strength of the adsorption solution? Are adsorbed antibodies desorbed by other proteins or by changes in pH or ionic strength? Can migrating protein be adsorbed to an antibody-containing strip? The model studies were done by adsorption of I-125 labelled transferrin, albumin and goat IgG to small circular pieces of the PVC-silica gel material as described in the Appendix.

The results showed that the pH of the adsorption solution influenced the amount adsorbed for all three proteins tested (Fig. 5). All three proteins had an adsorption maximum between pH 4 and 5. The amount of albumin and transferrin adsorbed decreased rapidly when the pH of the solution was increased. For IgG this decrease was very slow up to pH 9 where the adsorption curve falls rapidly. The amount adsorbed at a given pH was only slightly influenced by variations in the buffer concentration ( $\pm 10\%$ ) except for Tris-HCl buffer at pH 7.0 and 8.0. The adsorption in 1 mol/l Tris buffer was only 35 % of that obtained in the three lower concentrations tested.

The stability of the adsorption was examined by prolonged washing. After three or four washings the amount of radiolabelled protein in the washing solution was close to the background. Further washings with buffers of other pH did not remove more protein. Neither was any radioactivity released when one of the other two proteins was added to the washing solution. These results show that adsorbed proteins will not desorb when pH is changed to a value at which a smaller amount is adsorbed. Together with the adsorption profile obtained for albumin, this

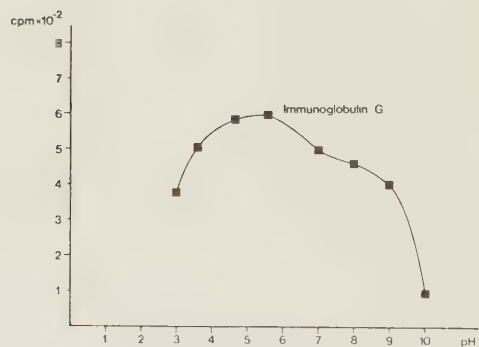
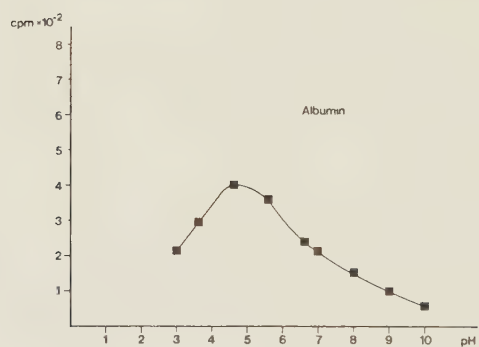
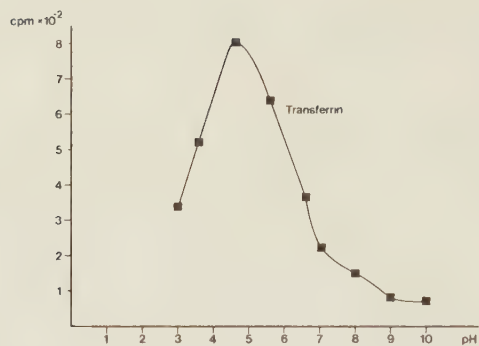


Fig. 5: Amount of radioactivity (cpm) adsorbed from a solution of  $^{125}\text{I}$  - labelled protein to circular PVC pieces as a function of the pH of the solution, when the buffer concentration was 0.1 mol/l.

is in accordance with results obtained by Norde and Lyklema (1978). They described the adsorption as being semireversible. When the pH was changed from where the adsorption was at a maximum no desorption was obtained. If the adsorption initially took place at a pH where the adsorption was low, followed by an adjustment of the pH to a value where the adsorption was maximal they obtained an additional adsorption. They also showed that the pH-dependent adsorption of albumin was nearly symmetrical with a maximum close to the isoelectric point of the protein, i.e. pH 4.5 - 4.8.

The PVC strips used in immunocapillarymigration were prepared and used in a 0.1 mol/l Tris-HCl buffer, pH 8.0. At this pH the amount of transferrin and albumin adsorbed is low compared to their maximum adsorption at pH 4.6. To make sure that no adsorption occurred during the capillarymigration radiolabelled albumin and transferrin were allowed to migrate using the two-step migration procedure described in Paper IV. In a strip with adsorbed non-immune goat IgG radiolabelled protein migrated in the first step and buffer in the second. After the second migration the labelled protein was only found in the upper third of the strip. Thus, the slower migration observed for transferrin in anti-transferrin containing PVC strips (I,II) was due to the specific interaction between antigen and antibody and not to non-specific adsorption of transferrin to the PVC material.

The PVC material had a high adsorption capacity. Approximately 1 mg protein was consumed per strip as estimated from the difference in absorption at 280 nm of the solution before and after incubation (II). About 80 % of this amount was adsorbed while 20 % was lost during the washing steps (unpublished results). The adsorption solution could be reused since only a fraction of the antibodies was consumed. Then the solution had to be concentrated since the adsorption is concentration dependent (unpublished results; Herrmann and Collins, 1976). The solution could be concentrated and reused twice before the amount of antibodies adsorbed started to decrease. The decrease was detected as an increase in the height of antigen-covered area produced by

migration of a standard sample compared to the height produced by the same sample in strips from the first or second preparation. That the adsorption was incomplete was shown by a high non-specific adsorption of fluorescein-labelled antibodies. A plausible explanation for this has not been found.

In all attachment procedures a purified IgG fraction of the antiserum has been used. In the text this IgG fraction has been referred to as antibodies, although only a small percentage is specific antibodies. This fraction has been purified in two different ways. The IgG fraction used in paper I, II and IV was prepared by ion-exchange chromatography of the antiserum and it was stored lyophilized. Lyophilization caused the IgG molecules to aggregate and the preparation contained a rather large amount (approximately 50 %) of dimeric IgG, shown by gel filtration (unpublished results). The IgG fraction used in paper III and V was prepared by affinity chromatography of a rabbit antiserum on Protein A-Sepharose. The IgG fraction was eluted with 0.1 mol/l glycine-HCl buffer, pH 2.2, and stored in 0.05 mol/l Tris-HCl buffer, pH 7.5, also containing sodium azide. Gel filtration of this IgG preparation showed that about 90 % was monomeric IgG (unpublished results). So far no studies have been performed to evaluate if the preparation method used influences the result obtained in immunocapillarymigration.

## CAPILLARYMIGRATION

Antibody-containing sheets can be used in immunocapillarymigration in two ways the sheet procedure and the strip procedure (Fig. 1 and 2; I). Both procedures are based on capillarymigration of antigen in an antibody-containing porous support. An important difference is that a defined amount of antigen is migrating for each sample in the sheet procedure, while the amount of migrating antigen is dependent on the height of migration of the solution in the strip procedure.

In the sheet procedure the height of the antigen-covered area obtained is dependent on the antigen concentration of the sample and the antibody concentration of the sheet. If the amount of attached antibodies in the strip is limiting, and therefore some antigen remain free the height of the antigen-covered area obtained will be equal to the height of migration (Fig. 6 A). If the height of migration is increased further the height of the antigen-covered area will also increase until all antigen molecules have bound to antibodies. An increase in height of buffer migration does not increase the height of an antigen-covered area for which the height in the first place was less than  $0.8 \times$  the height of the buffer-migration (Fig. 6; unpublished results). Thus, the highest concentration of antigen that can be determined for a given antibody concentration in the sheet is determined by the height of buffer-migration.

In the strip procedure, where the antigen is a constituent of the migrating solution, new antigen is entering the strip during the entire capillarymigration. The height of the antigen-covered area formed during the migration is therefore determined by the antigen concentration in the sample, the antibody concentration in the strip and how far the migration of the solution is allowed to proceed. To obtain quantitative results the migration height and the antibody content of different strips have to be kept constant.

During the application of several samples to a PVC sheet, the earlier applied samples will start to dry before the last one has been applied. This causes proteins in the samples to be non-specifically adsorbed at the application spot (unpublished observations). When several identical samples were applied on a sheet and the migration was allowed to start immediately after the last application, the different samples migrated differently. The sample applied first yielded a lower area covered with antigen than the last applied one. Reproducible results were not obtained unless all samples had been allowed to dry for at least 30 min. This is a drawback if the aim is to develop a rapid method. The non-specific adsorption is probably due to



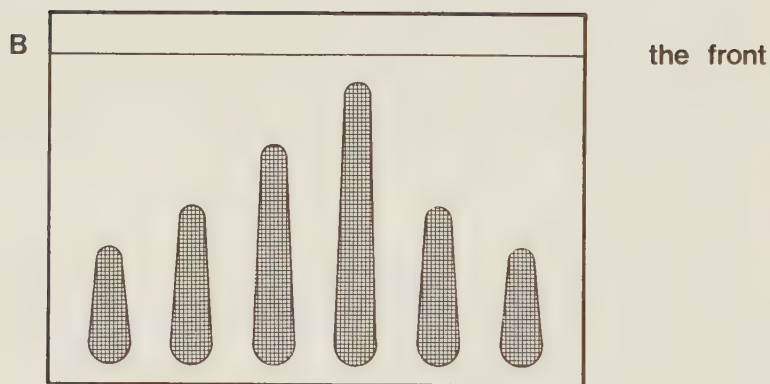
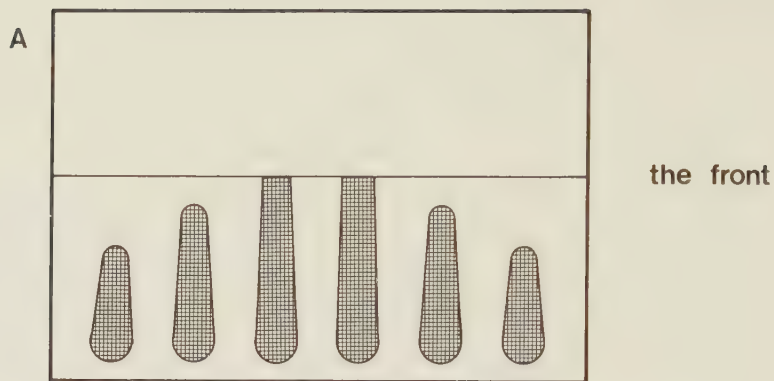


Fig. 6: A schematic drawing of a sheet after development of the antigen-covered areas. (A) when the migration was interrupted at the height of 30 mm and (B) when it was prolonged to a height of 60 mm.

denaturation of proteins. It is possible that the degree of denaturation may be affected by other factors as temperature, humidity, composition of the sample etc. which were constant in this investigation. Other porous materials than PVC in which the adsorption at the application point may be less have not yet been tested. It is also possible that the evaporation rate at the point of application can be decreased by additives, for instance poly(ethyleneglycol). When the two procedures are compared it is obvious that the sheet procedure is advantageous when a large number of samples are to be analyzed simultaneously.

A two-step migration procedure was used to characterize the immunocapillarymigration in the strips (IV). During the migration antigens were bound to antibodies in the strip, and the binding was rather stable. New antigen entering the strip during the migration, exchanged to a low degree with already bound antigen. It was also found that the concentration of antigen bound increased during the migration. This might be due to the longer time antigen and antibody are in contact during the later part of the migration, since the migration rate decreases with the distance of migration. Such an increased contact time may allow less accessible antibodies to react with antigen (IV).

It was found that the height of the antigen-covered area obtained for a particular antigen concentration was dependent on the migration rate of the sample (IV). Thus, a sample with a slower rate of migration gave a lower antigen-covered area than did a faster migrating sample. The lower area obtained is probably due to the longer contact time between antigen and antibody in this case. The rate of migration was found to be influenced by high protein concentrations in the sample. Undiluted serum had a slower rate of migration than had the same serum when diluted twice or more (IV). Consequently, undiluted serum samples could only be analyzed when all standard solutions used to obtain the standard curve had the same migration rate as the serum sample.



*Fig. 7: Immunocapillary migration strips after the antigen-covered areas have been exposed with peroxidase-labelled antibodies. The antigen concentration was not the same for both strips.*

The porous material must have good chromatographic properties for a uniform migration of the antigen in the immunosorbent. It is also important that the material is cut with a sharp knife or blade. If the edges of the sheets or the strips are frayed the migration will be uneven and the end of the antigen-covered area will be bent (Fig. 7). It is then difficult to measure the height of the antigen-covered area. The capillary migration of a sample in the PVC-material or in cellulose acetate is slow, approximately 60 mm per 30-45 min. Therefore the migration is performed in a humidified chamber to minimize evaporation from the sheets or strips (Fig. 1; II, III). In contrast the migration is rapid (60 mm per 5 min) in chromatography paper and a humidified chamber is not required (V).

#### DETECTION OF THE ANTIGEN-COVERED AREA

A useful detection method should have the following properties and requirements:

1. Specificity
2. Sensitivity
3. Be rapid and easy to perform.
4. No requirement for expensive equipment.
5. Give a visible area.
6. Give a sharp borderline of this area.

Specificity can be obtained either when a small amount of labelled antigen is added to the antigen-containing sample or when the obtained antigen-covered area is treated with labelled antibodies. In the first alternative an antigen-covered area containing both labelled and non-labelled antigen is obtained after the capillarymigration. In the second alternative one labelled antibody can be expected to have bound to each antigen molecule in the covered area. Hence, in the latter case most antigen molecules are indirectly labelled, while in the first case only a part of them are labelled, resulting in a lower sensitivity. Therefore, labelled antibodies were used for exposure although this procedure is more laborious.

In the work done so far on immunocapillarymigration the sensitivity of the method is limited by the detection system. When the amount of antibodies in the strip is decreased the antigen-covered area will contain a lower concentration of antigen and hence a lower concentration of labelled antibodies is bound to this area. For each kind of label there will be an antigen concentration below which the label can not be detected. The lowest antigen concentration detected using fluorescein isothiocyanate (FITC) has been 40 mg/l (I,II). At lower antigen concentrations the intensity of the fluorescent area became so low that the boundary of this area could not be monitored with acceptable precision. The mean FITC:protein (F/P) ratio was 4.0 in this system (II). A higher F/P ratio did not improve the sensitivity since such conjugates were adsorbed non-specifically to the strip increasing the background fluorescence (unpublished results). An attempt to increase the sensitivity by purifying the conjugates used (Forsum, 1973) was unsuccessful.

To improve the sensitivity of the method other labels than FITC had to be found. Enzyme labels are potentially very powerful since the product of the enzyme-catalysed reaction can be used for detection. Thus, the sensitivity can be improved considerably by utilizing the amplification obtained in the catalyzed reaction.

A suitable enzyme label for use in immunocapillarymigration has to meet several requirements. Most of them are the same as those set up for enzymes used in enzyme-immunoassays (Wisdom, 1976; Mattiasson and Borrebaeck, 1980). In addition, the enzyme has to catalyze a reaction producing a coloured insoluble product which precipitates where the enzyme is located in the strip. Horse-radish peroxidase has been used in enzyme-immunoassays (Wisdom, 1976; Mattiasson and Borrebaeck, 1980), but in combination with substrates that give soluble products. The enzyme also catalyzes the oxidation of 3-amino-9-ethylcarbazole yielding a water-insoluble red product (Graham et al. 1965). Peroxidase-labelled second antibodies have been used with this substrate for amplification of the precipitates formed in electroimmunoassay and single radial immunodiffusion (Ingild, 1981; Löfberg and Grubb, 1979). This enzyme-substrate combination was tried in immunocapillarymigration. It improved the sensitivity and the precision of the method compared to when FITC-labelled antibodies were used (III). Concentrations of antigens down to 0.15 mg/l could be quantified with a precision of 7 %.

The two most commonly used techniques for conjugation of peroxidase to antibodies are the two-step glutardialdehyde method (Avrameas and Ternynck, 1971) and the periodate technique (Wilson and Nakane, 1978). We have tested both these techniques and obtained a high degree of conjugates with 1-2 peroxidase molecules/antibody with either method. The stoichiometry of the conjugates was determined by the  $A_{403}/A_{280}$  ratio obtained in the conjugate peak after chromatography on Sephacryl S-200 (Wilson and Nakane, 1978) (Fig. 8). The periodate technique was more efficient than the glutardialdehyde one, with 75 % of the added peroxidase conjugated compared to 60 % using



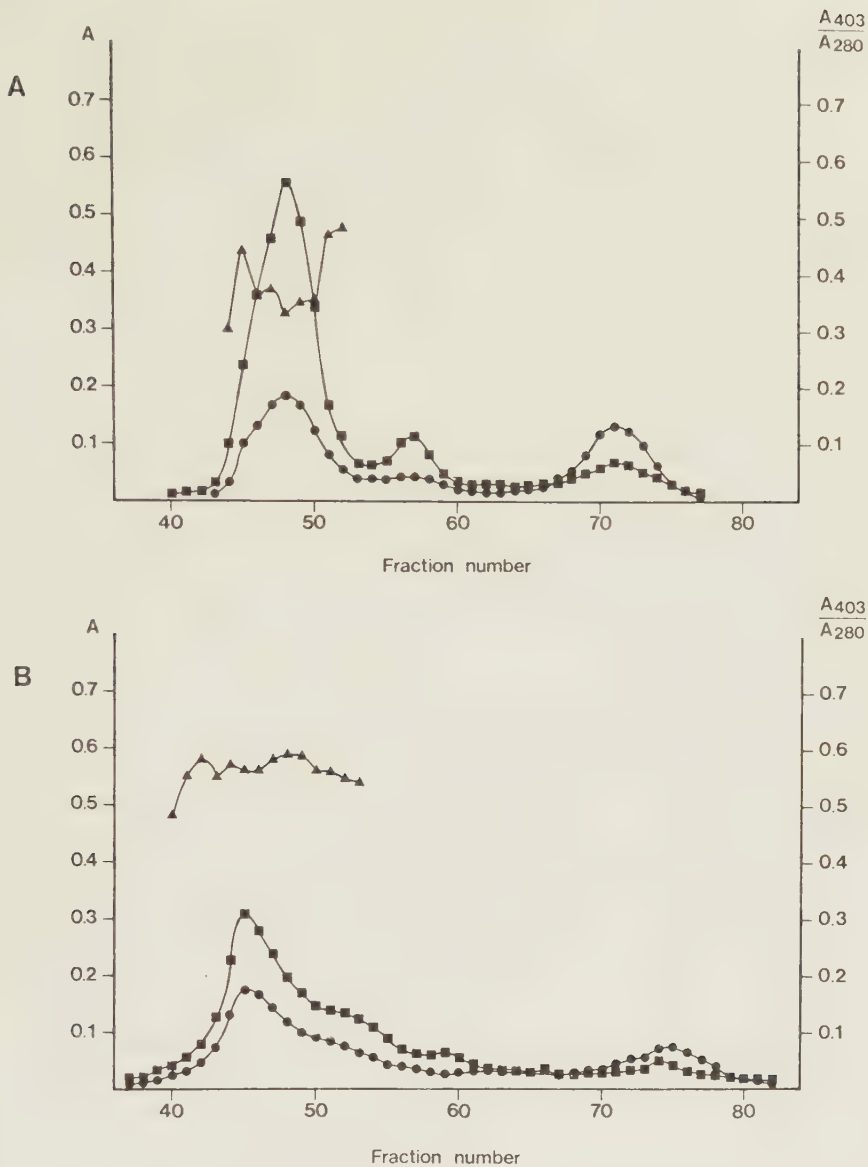


Fig. 8: Separation of horse-radish peroxidase-IgG conjugation mixtures on a Sephacryl S-200, column size: 2.5 · 80 cm. Elution buffer 0.05 mol/l Tris-HCl, pH 7.4, containing 0.5 mol/l NaCl. The fraction volumes were 3 ml. (A) conjugation mixture obtained with the two-step glutardialdehyde method and (B) with the periodate technique. ■,  $A_{280}$ ; ●,  $A_{403}$ ; ▲,  $A_{403}/A_{280}$ .

glutardialdehyde (Fig. 8). There was also a difference between the conjugates in the time required for a heavily stained area to develop. Conjugates prepared using periodate required only half the time of conjugates prepared using glutardialdehyde. The much longer time required for the latter conjugates resulted in a more diffuse demarkation of the stained area and thus a lower precision (Glad and Grubb, 1980; III).

The enzyme activity of the different conjugates has been studied with the substrate 4-aminoantipyrine (Lott and Turner, 1975). This study showed a higher activity for conjugates prepared by periodate coupling (unpublished results). Whether this difference in enzyme activity was the only reason for the lower catalytic activity observed on the antigen-covered area, or if there was also a difference in the amount of labelled antibodies bound has not been studied.

When low antigen concentrations were quantified the concentration of labelled antibodies specifically bound to the antigen-covered area was also low. The lowest concentration of peroxidase-labelled antibodies that could be detected was dependent on the concentration of labelled antibodies non-specifically bound to the strip. If the difference in concentration of specifically and non-specifically bound antibodies was small, the boundary of the antigen-covered area was difficult to recognise. With an efficient washing step following the incubation with labelled antibodies the amount of non-specifically bound antibodies could be reduced to a very low level improving the sensitivity of the procedure. However, as the amount of labelled antibodies was decreased an increased period of incubation was required to get a densely coloured area. As mentioned above, such an increase in time results in a diffuse demarkation and thus a lower precision. For very low concentrations of enzyme-labelled antibodies on the area an unacceptable precision will be obtained. Thus, the sensitivity obtained using peroxidase-labelled antibodies was determined by the amount of labelled antibodies that was adsorbed non-specifically to the strip and by the incubation time required to obtain a heavily stained

area.

In the beginning of this section some requirements for an optimal detection procedure were listed. So far no procedure has been found that fulfil all requirements. The use of peroxidase-labelled antibodies is advantageous since it is a specific and sensitive detection method which is rather rapid (10 min), no special equipment is required and the stained area is readily visible. The drawback of the method is that four steps are added to the immunocapillarymigration procedure (Fig. 2). Three of these steps will be required for any detection method in which labelled antibodies are used. The first washing step can not be avoided since the antigen-covered area contains both free and bound antigen (IV). If the free antigen is washed off during the incubation with labelled antibodies these would be consumed rapidly. If the second washing step is omitted the strip will contain an excess of labelled antibodies when incubated in the substrate solution and the entire strip will be heavily stained.

The most simple detection procedure should not require any additional handling after the capillarymigration. This can be obtained if labelled antigen is added to the sample to be examined and the label is directly visible. The problem is that the label has to be detected at extremely low concentrations if the method should be sensitive enough. This is illustrated by the following calculation which is made for a strip used for quantitation of C-reactive protein (CRP) (MW 120 000) down to a level of 0.15 mg/l (1.25 nmol/l) (III):

A cellulose acetate strip contains 50  $\mu$ l of the sample solution when the migration is interrupted at the height of 60 mm. If the sample solution contains 0.15 mg CRP/l (1.25 nmol/l) the total amount of antigen in the strip is 7.5 ng (62.5 fmol) before washing. Two antigen-covered areas, one on each side of the strip, 5 mm wide and 13 mm high, are obtained. The bottom 5 mm of the strip inserted in the solution, has been

included. If it is assumed that the antigen is only present at the surface of the strip, the antigen concentration on the covered areas will be  $58 \text{ pg/mm}^2$  or  $0.48 \text{ fmol/mm}^2$ . This is the total antigen concentration, only a small fraction of the antigen is labelled, and so far no label has been found that is directly visible at such low concentrations.

#### QUANTITATIVE DETERMINATION

Immunocapillary migration can be used for quantitative purposes if uniform coupling of antibodies to the porous material can be obtained. With the attachment procedures previously discussed it has been possible to prepare several sheets at the same time which all have the same amount of antibodies uniformly coupled. When these sheets are used in either the sheet or strip procedure the size of the antigen-covered area obtained is related to the concentration of antigen in the sample. The area will increase as the antigen concentration increases. In practice the area has been assumed to be proportional to the height of the area. This is true if the area is symmetrical and the height is measured along the axis of symmetry as has been found in the sheet procedure when symmetrical 'rocket'-shaped areas were seen after a uniform migration. In the strip procedure the boundary between covered and non-covered areas are usually meniscal since the migration at the edges is more rapid than in the middle of the strip. If the strips are cut sharply the antigen-covered area will have a symmetry line along the middle of the strip and the height at the centre can be used for an accurate quantitation. If the edges of the strip are frayed the migration will be uneven (Fig. 7) and the height at the centre of the area can not be used for quantitation.

Since quantitative studies have been performed primarily by using the strip procedure (I-V) this will be discussed in the following.

The relation between the height of the antigen-covered area and the concentration of antigen in the sample is not linear (II, IV, V). The following calculation shows that a linear relation does not exist even in an idealized case. The calculation is based upon the following assumptions: all antibodies are equally accessible and the antigen-antibody reaction is instantaneous, irreversible and independent of the antigen concentration.

In a strip the front has migrated to the height of  $y$  mm. An antigen-covered area  $h$  mm high has been obtained and the solution volume contained in the strip is  $v$  mm<sup>3</sup>. Thus the volume contained in a volume element 1 mm high is  $A = \frac{v}{y}$ . The antigen concentration in the sample is  $c$  mg/mm<sup>3</sup> and the antigen concentration that is bound is  $a$  mg/mm<sup>3</sup>.

After the migration the strip can be divided in an antigen-free part  $(y - h)$  mm high and an antigen-covered part  $h$  mm high. The solution volume in the antigen free part is  $A \cdot (y - h)$ , and it contained originally  $c \cdot A \cdot (y - h)$  mg antigen that is now bound to the antigen-covered part. The amount of bound antigen in the latter is  $a \cdot h \cdot A$ . With the assumptions made above the following relation is obtained:

$$c \cdot A \cdot (y - h) = a \cdot h \cdot A \quad (1)$$

which can be rearranged:

$$h = \frac{y \cdot c}{c + a} \quad (2)$$

For strips with the same concentration of antibodies is constant, and the height is a function of the concentration. This functional relationship is not linear (Fig. 9).

If experimentally obtained values of  $h$  and  $c$  for strips known to contain the same amount of antibodies are inserted in equation (2) the calculated values for  $a$  indicate the difference between



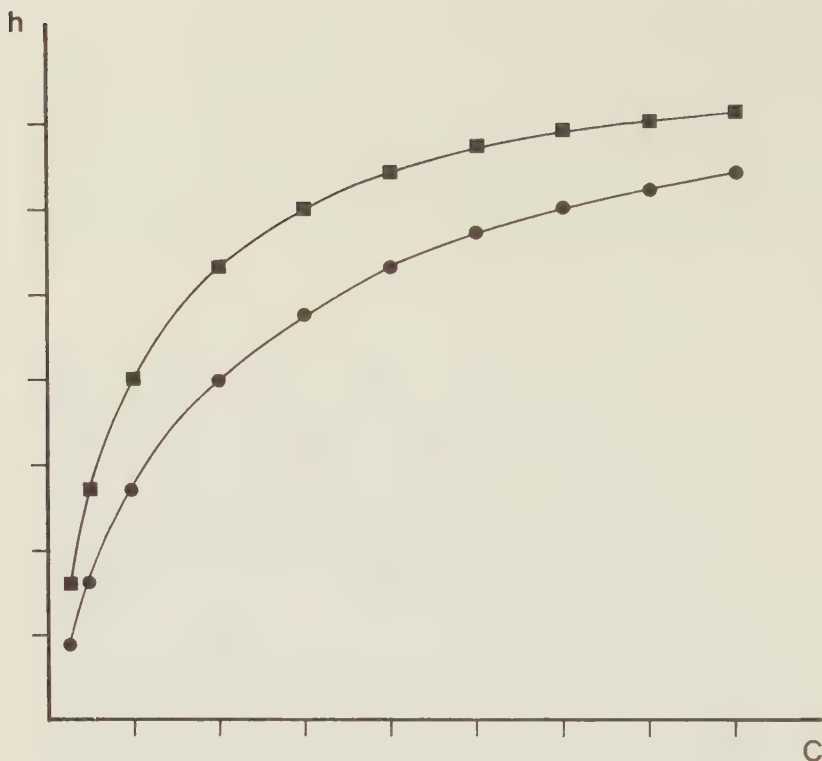


Fig. 9: A graphic representation of the mathematical relation  $h = \frac{y \cdot c}{c + a}$  for two values on  $a$ ; ■ ,  $a=1$  and ● ,  $a=2$ ;  $y=8$ .

the real case and the ideal. Such values are shown in Table I where experimentally determined  $h$  and  $c$  from the standard curves of Fig. 2 in Paper IV have been used to calculate  $a$  values. As can be seen the values increase with increasing antigen concentration. Hence, in practice  $a$  is a function of both the concentration of specific antibodies in the strip and the antigen concentration.

As was discussed under Capillary migration the migration distance should be kept constant to obtain quantitative measurements. We have used a height of 60 mm (I-V). This has been chosen due to two methodological factors. One is the time required for migration which should be kept as short as possible.

TABLE I

Calculated values for  $\alpha$ , the binding capacity of a strip obtained from experimentally related values of antigen concentration,  $c$ , and height of covered area,  $h$  (see text).

| Antibody concentration in strips | Antigen concentration (mg/l) | Height of covered area (mm) | $\alpha$ (mg/l) |
|----------------------------------|------------------------------|-----------------------------|-----------------|
| C                                | 20                           | 31.5                        | 18.1            |
|                                  | 10                           | 22.5                        | 16.6            |
|                                  | 5                            | 14.5                        | 15.7            |
|                                  | 2.5                          | 10                          | 12.5            |
| C/2                              | 20                           | 41.5                        | 8.9             |
|                                  | 10                           | 32.5                        | 8.5             |
|                                  | 5                            | 22.5                        | 8.3             |
|                                  | 2.5                          | 15                          | 7.5             |
|                                  | 1.25                         | 10                          | 6.3             |

The migration rate decreases with height, and it was found that an increase in the height from 60 to 65 mm in PVC or cellulose acetate strips increases the time required with 8 - 10 min (unpublished results). The other factor is the slope of the standard curve. When the migration distances are increased the slope of the standard curve will also increase and smaller differences in antigen concentration can be detected (unpublished results). This effect is also described by equation (2). The migration height of 60 mm was an acceptable compromise for both factors.

When the concentration of attached antibodies in the strip is decreased the amount of antigen that can be bound per square unit also decreases. Lower concentrations of antigen can thus be determined. To describe the relation between the height of the antigen-covered area, the antibody concentration in the strip and the antigen concentration in the sample, the model described above can be used. If equation (2) is applied for two strips with the antigen binding capacities  $\alpha$  and  $\frac{\alpha}{2}$  the curves of Fig. 9 are obtained. If it is assumed that the antibody concentration

in a strip is proportional to its antigen binding capacity, the curves can be used to compare two strips that differ in antibody concentration with a factor of 2. It is seen that when the antibody concentration is doubled the antigen concentration also has to be doubled to obtain the same height of the antigen-covered area on the two strips. This is in agreement with the results obtained when this relation was studied experimentally (IV). In practice this means that when a preparation of sheets have been obtained and the antigen concentrations it covers has been identified from a standard curve, that knowledge can be used for preparations of new sheets for use at a desired concentration level of antigen. If the coupling procedure is the same such sheets are obtained by diluting the coupling solution with a solution of non-immune IgG from the same species. The dilution factor is then equal to the factor with which the antigen level should be decreased (IV).

So far monospecific antibodies have been used both in the porous material and in the detection step (I-V). The method will be specific as long as monospecific antibodies are used in one of these steps. Thus, if a serum sample is analyzed with a strip containing antibodies against several serum proteins, the specificity of the labelled antibodies used for detection will determine which protein that is quantified. The relation between antibody concentration in the strip and the concentration level of antigen which can be quantified has been used to prepare a strip that in the same serum dilution can determine either the IgG,  $\alpha_1$ -antitrypsin or the transferrin concentration (unpublished results).

## H I G H     V O L T A G E

### E L E C T R O I M M U N O A S S A Y

Electroimmunoassay (Laurell, 1966; 1972) is a relatively rapid method for immunochemical quantitation of proteins. It is based upon electrophoresis of an antigen in an antibody-containing agarose gel. The antigen-containing samples are applied in small wells in the agarose gel and an electric field is applied over the gel. When the antigen migrates into the gel it will bind to the antibodies, which are stationary in the gel-buffer used. Rocket-shaped precipitates are formed that are proportional in area to the amount of antigen in the sample. The time required for a precipitate to develop under standardized experimental conditions varies from antigen to antigen. It is dependent on the migration rate of the antigen in a gel without antibodies and on the retardation caused by the immunochemical reaction. The time required for determination of most plasma proteins in 0.075 mol/l barbital buffer, pH 8.6, and a field strength of 10-15 V/cm varies from 3 to 8 h. Attempts to decrease this time have been made (Renn and Evans, 1976) by using a buffer of lower ionic strength, but no systematic study concerning the relation between the ionic strength, field strength and the time required to complete an assay has been published. Paper VI presents such a systematic study for transferrin and the application of the results to other plasma proteins.

The most obvious way to increase the migration rate of an antigen, thereby decreasing the time required for the formation of a precipitate, is to increase the strength of the electric field applied over the gel. According to the relation

$$W \text{ (watt/ml)} = \frac{E \cdot i}{A}$$

where  $E$  is the field strength,  $i$  is the current, and  $A$  is the cross-sectional area of the gel, such an increase also results in an increase of the heat produced during the electrophoretic run. With insufficient cooling the temperature of the gel will increase leading to a destruction of the homogeneity of the electric field (Wieme, 1965). This effect of the electric heating can be balanced by reduction of the current. The most practical method to decrease the current is to use a buffer of lower ionic strength or electrolytic conductance.

From Debye and Hückels work (Debye and Hückel, 1923; Hückel, 1924) on the mobility of an electrically charged particle in an ionic environment the following relation will be obtained for the mobility,  $\mu$ , at unit field strength

$$\mu = \frac{q}{6 \cdot \pi \cdot \eta \cdot r} \cdot \frac{1}{1 + \kappa \cdot r}$$

where  $\kappa$  is the Debye-Hückel parameter which is a function of the square root of the ionic strength at constant temperature,  $q$  is the charge of the particle,  $r$  is the radius and  $\eta$  the viscosity of the solution. The given relation is only valid for strong electrolytes or for completely ionized weak electrolytes, and thus not for ampholytes as proteins. It shows, however, that there is an inverse relation between the mobility and the ionic strength of the medium. Hence, if the current is reduced by decreasing the ionic strength the migration rate of the protein will be increased. There is a limit, however, to how much the ionic strength can be reduced, set by the decrease in buffering capacity and the solubility of the proteins at low ionic strength. In addition, the electroosmotic flow in the gel is governed by roughly the same laws as the migration of proteins. A decrease in ionic strength will thus increase the osmotic flow and antibodies may be carried away from the gel.



The discussion above is based upon theories derived for a spherical, fully ionized particle with the charge uniformly distributed over its surface. These ideal conditions do not apply in electrophoresis of proteins or in electroimmunoassay when the antigen is bound to an antibody during the electrophoretic run. Hence, in paper VI the relation between ionic strength, electric field strength and the time for a transferrin 'rocket' to be fully developed was studied experimentally. The system examined is complex and the relations obtained include effects on migration of transferrin and transferrin-antibody complexes, effects on the immunochemical reaction and on precipitation. Thus, only a descriptive evaluation of the results was possible.

The investigation showed that both an increase in ionic strength and in the electric field strength decreased the time required for a quantitation (VI). At high field strengths ( $> 43$  V/cm) the inhomogeneity of the electric field around the application wells became obvious, however. If samples were diluted with buffer only, the wells were quickly drained and the gel dried between the wells resulting in a break of the current. A more homogeneous field was obtained when samples were diluted with an agarose solution. Then a field strength of 62 V/cm could be used. A combination of this field strength with a low ionic strength, 0.01 mol/l barbital buffer, pH 8.6, reduced the time required for a transferrin precipitate to be formed to 20 min. The system was successfully applied for other plasma proteins and quantitative results were obtained within 9 min for albumin and within 25 min for IgG.

## Q U A N T I T A T I V E   C R O S S E D

## I M M U N O E L E C T R O P H O R E S I S

Crossed immunoelectrophoresis (Laurell, 1965; Ganrot, 1972) is distinguished by its high resolving power and the possibility to demonstrate antigen heterogeneity, complex formation and polymorphism of antigens in the sample. It comprises two experimental steps, the separation of antigens, and the immunochemical reaction between the separated antigens and the antibodies. The separation is achieved by electrophoresis in an agarose gel (Johansson, 1972). A gel strip containing the separated antigens is cut out and transferred to a slit (of the same size as the strip) in an antibody-containing gel. An electric field is applied over the antibody-containing gel perpendicular to the length axis of the gel strip. As in electroimmunoassay (see previous section) the antigen will migrate into the gel and react with antibodies to form areas enclosed by precipitates.

As described by Laurell (1965) the crossed immunoelectrophoresis is only semi-quantitative. The gel strip from the first gel does not contain the total amount of proteins applied. In Clarke and Freeman's modification of the technique (Clarke and Freeman, 1967; 1968) all antigen applied in the first dimension is transferred to the second dimension. This is achieved by applying the sample in a circular well and by transferring the whole first-dimension gel. A quantitative method where the peak-height of the precipitates is proportional to antigen concentration is thus obtained. The separation in the first step is, however less efficient than in the Laurell version.

Several plasma proteins are known to form complexes with other proteins (Laurell, 1970; Laurell and Thulin, 1975; Tejler and Grubb, 1976) and may thus occur in several molecular forms in the plasma. Even if all molecular forms react with a mono-specific antiserum the stoichiometry of the reaction may not be the same. An accurate quantitative determination of such proteins may therefore be difficult to perform with conventional immunochemical techniques. Separation of the different forms and individual quantitative determinations with standards of the same molecular form gives the most correct result.

The different forms usually vary in electrophoretic mobility and this heterogeneity can be demonstrated by crossed immunoelectrophoresis (Ganrot, 1972). The application of a quantitative crossed immunoelectrophoretic technique would thus render the rapid and simple separation and quantitation of different forms of a protein possible. The low resolving power of the Clarke and Freeman procedure makes it unsuitable for such determinations.

A quantitative version of the Laurell type of crossed immunoelectrophoresis is described in paper VII. The salient feature of this modification is that an internal standard is added to the sample to monitor variations in the amount of sample that is transferred to the second step. It is important that the internal standard does not form complexes with any of the proteins in the sample or that it crossreact with the antiserum used. We have used ovalbumin as internal standard since it is not likely to interact with mammalian serum proteins. The ovalbumin was reduced and carboxymethylated before use to further reduce the possibilities for interaction with sulphydryl groups or disulphides of the sample proteins.

## C O N C L U S I O N

A comparison of the three described methods for immunochemical quantitation of proteins shows that they are complementary to each other.

Immunocapillarymigration is characterized by its simplicity, rapidity and its independence of technical equipment. These features suggest applications in acute situations and under field conditions. The technique is not fully developed and several characteristics have not been studied yet. Further work will be directed towards characterization and optimization of the technique for use under different field conditions.

Electroimmunoassay is a well developed and characterized technique, routinely used in many clinical laboratories. It is less suited for use outside the laboratory, since trained technicians and technical equipment is required. In the described high voltage modification the method is highly competitive with nephelometric methods. The technical equipment required is simple and inexpensive and mono-specific antisera are not needed. In addition, information about the quantitated antigen can be obtained by inspection of the precipitates.

Crossed immunoelectrophoresis in the Laurell version has an established position in research on protein heterogeneity. In the modified form described here it enables rapid quantitation of different molecular forms of a protein. The modified technique is much less laborious than conventional methods including chromatographic separations for quantitation of various molecular forms of an antigen.

## A P P E N D I X

### MATERIALS

Pure human transferrin and albumin and goat IgG were available at the laboratory. Polyvinyl chloride sheets were purchased from Amerace Erna Corporation, Butler, N.J., USA.

Buffers: Four concentrations, 1.0, 0.1, 0.05, and 0.01 mol/l, of each of the following buffers were used:

|                  |         |
|------------------|---------|
| glycin-HCl       | pH 3.0  |
| sodium acetate   | pH 3.6  |
| sodium acetate   | pH 4.6  |
| sodium phosphate | pH 5.6  |
| sodium phosphate | pH 6.5  |
| Tris-HCl         | pH 7.0  |
| Tris-HCl         | pH 8.0  |
| glycin-NaOH      | pH 9.0  |
| glycin-NaOH      | pH 10.0 |

### METHODS

Preparation of iodinated proteins: Human transferrin and albumin and goat IgG were labelled with I-125 according to Thorell and Johansson (1971).

Adsorption studies: Circular pieces, 5 mm in diameter, were punched out of the PVC material and were washed with distilled water. Five pieces were incubated for 18 h at room temperature in 2 ml of a solution containing 5 mg/ml of a protein and 10  $\mu$ l of a solution of I-125 labelled protein. The tubes with the incubation mixture were constantly agitated. After the incubation



the pieces were washed with 5 x 10 ml of the same buffer as was used in the adsorption solution. The amount of adsorbed protein on each piece was measured in a gamma counter.

## T A C K .....

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## A New Method for Immunochemical Quantification

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A large number of methods for immunochemical quantification have been developed during the last 50 years. To bring about the necessary interaction between antigen and antibody these methods use either diffusion, as in single radial immunodiffusion (Mancini *et al.*, 1965), an electrophoretic process, as in electroimmunoassay (Laurell, 1966), or mixing as in radioimmunoassay (Ekins, 1960; Yalow & Berson, 1960) and nephelometry (Ritchie *et al.*, 1973). This report demonstrates that the capillary force in a porous material can also be used to bring about an antigen-antibody interaction and that this can be used for quantitative purposes.

Antibodies were attached to porous insoluble supports, such as cellulose acetate sheets (Sartorius, Göttingen, W. Germany), filter paper Munktell 00 (Glycksbo Pappersbruk, Sweden) and polyvinyl chloride sheets containing silica gel as a filler (Amerace Erna Corporation, New York, NY, U.S.A.). Methods used for attaching antibodies were either covalent linkage with CNBr (Nishikawa & Bailon, 1975) or cross-linking with glutardialdehyde for cellulose acetate and filter paper, and adsorption from 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.5% (w/v) goat or rabbit antibodies for polyvinyl chloride sheets. The antibody-containing porous sheets were then used in two different ways. In the first procedure, 1–5  $\mu$ l of sample solutions was applied on a line 1 cm from the edge of a sheet, and the sheet was placed in a chromatographic chamber with 5 mm of it inserted in 0.1 M-Tris/HCl, pH 8.0, and the capillary migration of the buffer was allowed to proceed upwards. When the buffer had migrated 6 cm, the sheet

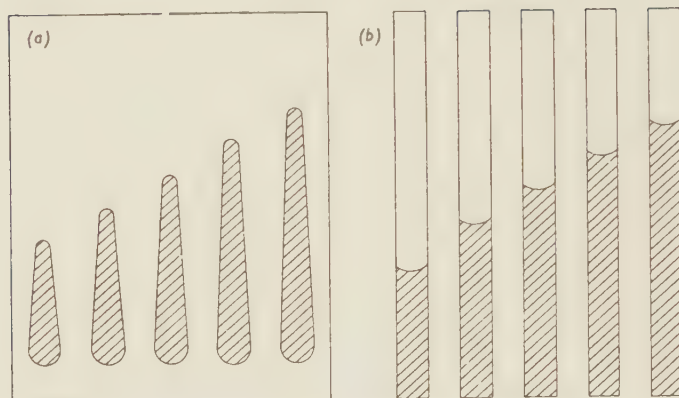


Fig. 1. Drawings showing fluorescent antigen-covered areas on a sheet (a) and on strips (b) containing antibodies

The fluorescent areas were produced by applying antigen solutions of increasing concentrations to the antibody-containing materials, allowing a capillary migration to take place, and then immersing the material in a solution of fluorescein-conjugated antibodies against the antigen.

was removed and the antigen-covered areas made visible by incubating the sheet in a solution of fluorescein-labelled antibodies for 3 min, washing off excess fluorescein-labelled antibodies under running tap water and finally inspecting the sheet in u.v. light (wavelength 254 or 350 nm). The fluorescent areas were marked by a pencil (Fig. 1a).

In the second procedure the antibody-containing sheets were cut into strips of 8 mm × 70 mm. Each strip was placed in a cup, 9 mm in diameter, containing 300 μl of a sample dilution, and the cups were placed in a closed humidified chamber. The sample solution was allowed to migrate to a height of 6 cm. The strips were then removed from the cups and excess antigen solution was washed off the strips under running tap water for about 2 min. The antigen-covered area of each strip was then exposed and marked as described above (Fig. 1b).

The heights of the fluorescent areas produced by the samples in both procedures were compared with the heights produced by serial dilutions of a standard. For sheets and strips of the same material containing the same amounts of attached antibodies the heights of these areas were found to increase with increasing amount of antigen in the sample (Figs. 1 and 2).

When the performances of cellulose acetate and filter paper in these immunochemical procedures were compared with that of the polyvinyl chloride material, several disadvantages were associated with the first two materials. Antibodies had to be covalently linked to the materials and it was difficult to devise a method for reproducible attachment of a specified amount of antibodies. The materials also showed a considerable non-specific binding of fluorescein-labelled antibodies and were very fragile. The polyvinyl chloride sheets showed none of these drawbacks.

The two different procedures described were both easy to perform and both procedures required 45–60 min. The sheet method requires smaller sample volumes and is easy to handle when a large number of samples are to be simultaneously analysed. A disadvantage of this procedure is that a limited amount of non-specific binding of antigen at the application takes place. For the strip method there are no problems associated with the sample application, but the capillary migration must be interrupted at exactly the same height for both sample and standard solutions.

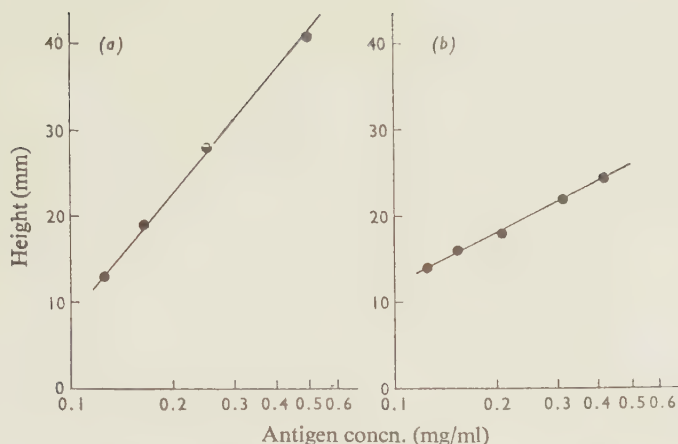


Fig. 2. Standard curves relating the heights of the fluorescent areas obtained on a sheet (a) and on strips (b) and the antigen concentration of the applied solutions (logarithmic scale)

A comparison of the strip method and single radial immunodiffusion in the quantification of transferrin in 29 samples of human sera gave a correlation coefficient of 0.91. The standard deviation of the method calculated from duplicate determinations was found to be about 10% of the mean for both high- and low-range concentrations (C. Glad & A. O. Grubb, unpublished work).

When the antibody activity of the immunoglobulin fraction attached to the materials was decreased, the fluorescent area produced by migration of one and the same sample increased, with concomitant decrease in intensity of fluorescence. Our results for the two procedures described indicate that their sensitivity is about  $50 \mu\text{g/ml}$  when the system for antigen detection reported in this work is used.

Since both procedures for immunochemical quantification described here utilize antigen-antibody interactions brought about by capillary migration of solutions, they can be considered as special cases of a more general procedure for immunochemical quantification. For this general procedure we suggest the term immunocapillary migration.

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## Immunocapillarymigration—A New Method for Immunochemical Quantitation

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A new simple and rapid method for immunochemical quantitation called immunocapillarymigration is described. It is based upon the attachment of antibodies to a porous insoluble support and the subsequent capillarymigration of the antigen-containing solution in the porous support. The migration of the antigen solute is specifically delayed in comparison to the migration of the solvent and other solutes in the process and the relative delay decreases with increasing antigen concentration. When applied to the quantitation of transferrin in human plasma, immunocapillarymigration gave results which agreed with those obtained by single radial immunodiffusion.

Since the first quantitative immunochemical principles were established by Heidelberger and Kendall (1) some 50 years ago, a large number of methods for immunochemical quantitation have been developed. Some of these methods, e.g., single linear diffusion according to Oudin (2), single radial immunodiffusion according to Mancini *et al.* (3) or according to Fahey and McKelvey (4), double linear diffusion according to Preer (5) or Ouchterlony (6) or according to Wieme and Veys (7), Farr's ammonium sulfate technique (8), Laurell's electroimmunoassay (9), automated nephelometric analysis (10), and various types of radioimmunoassays (11,12) are presently used in most laboratories. Some of the above-mentioned methods are distinguished by simplicity and rapidity while others are distinguished by extremely high sensitivity. All the methods employ either mixing, diffusion, or an electrophoretic process to bring about the necessary interaction between antibody and antigen. In this work we present a new simple and rapid quantitative immunochemical method in which capillary force is used to bring about the necessary antigen-antibody interaction.

### MATERIALS

Polyvinylchloride sheets containing silica gel as a filler (microporous plastic sheets) were purchased from Amerace Erna Corporation, New York, N. Y., fluorescein isothiocyanate from BDH Chemicals, Pool,



England, and Sephadex G 25 from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of reagent grade. Pure human IgG, albumin, and transferrin were available at our laboratory.

*Preparation of antisera.* Goat and rabbit antisera against human IgG, albumin, and transferrin were produced by repeated subcutaneous injections of the purified antigens emulsified in Freund's complete adjuvant. Sera from several bleedings were pooled and the pools were rendered monospecific by appropriate absorptions. Immunoglobulin fractions of the antiserum pools were produced by ammonium sulfate precipitation. The precipitates were dissolved in and dialyzed against distilled water, pH 5–6, and then lyophilized.

*Preparation of fluorescein-labeled antibodies.* Parts of the immunoglobulin fractions of the antisera were labeled with fluorescein isothiocyanate (13). The fluorescein-labeled immunoglobulins were freed of small molecular weight contaminants by gel chromatography on Sephadex G 25 in 0.1 M Tris-HCl buffer, pH 8.0, containing 8 mmol/liter of sodium azide. Aliquots of the solutions of fluorescein-labeled immunoglobulins were stored at  $-20^{\circ}\text{C}$  until used. The mean fluorescein:protein molar ratio of the conjugates was measured as described by Brandtzaeg (14) and was about 4.0 in the various preparations. The protein concentration of the solutions was determined spectrophotometrically (14) and was found to be about 1%.

## METHODS

*Single radial immunodiffusion.* The procedure of Mancini *et al.* (3) was followed. A diffusion period of 48 hr was used, since no further increase of the precipitate areas occurred after this time.

*Attachment of antibodies to capillary-containing sheets.* Microporous plastic sheets were washed for 3 hr in three changes of 0.1 M Tris-HCl buffer, pH 8.0, containing 8 mmol/liter of sodium azide and excess buffer was expressed from the material using filter paper. The porous material was then immediately incubated for 18 hr at  $4^{\circ}\text{C}$  in the above-mentioned Tris buffer containing 0.5 g/100 ml of goat immunoglobulins against human transferrin. After the incubation the sheets were washed by the procedure described above. The sheets were then dried between filter papers and left to equilibrate with the air humidity for a week in darkness at room temperature. Strips measuring  $70 \times 8$  mm were then cut with a very sharp blade from the antibody-containing sheets and a mark was made at the height of 60 mm on each strip.

*The immunocapillary migration procedure.* The samples to be analyzed were diluted with 0.1 M Tris-HCl buffer, pH 8.0, containing 8 mmol/liter of sodium azide and about 300  $\mu\text{l}$  of the dilutions were poured into cylindrical cups with a diameter of about 9 mm and the cups were placed in a closed humidified chamber. One end of an antibody-containing strip was then

placed in each cup and the other end was allowed to lean against one of the walls of the chamber. By this procedure an equal portion of each strip was submerged in the sample solution. The solution started thereby to migrate up the strip by capillary force and when it had reached the mark on the strip, after about 45 min, the strip was removed from the cup and excess antigen solution was washed off the strip under running tap water for about 2 min. The antigen-covered area of each strip was then detected by incubating the strip for about 3 min in the above-mentioned Tris-HCl buffer containing fluorescein-labeled antibodies against the antigen of interest. Excess fluorescein-labeled antibodies were washed off the strip under running tap water as described above. The strip was then inspected in ultraviolet light (wave length, 254 nm) and the fluorescent area was marked by a pencil. The antigen concentration in each serum sample was then obtained by comparing the height of the fluorescent area produced by the serum sample with corresponding heights produced by serial dilutions of a standard serum.

## RESULTS

If a serum sample is allowed to migrate by capillary force in a porous strip with adsorbed anti-transferrin antibodies and the strip is then washed and incubated for a short time in a solution of fluorescein-labeled antibodies against human transferrin the strip will show a bright fluorescent area (Fig. 1A). For strips containing the same amounts of adsorbed antibodies the height of this area was found to be proportional to the logarithm of the transferrin concentration in the sample (Figs. 1A and B).

*Specificity.* When rabbit or goat IgG from nonimmune sera was adsorbed to a porous strip and human serum was allowed to migrate in the strips no fluorescence could be demonstrated on the strips by means of fluorescein-labeled rabbit and goat antibodies against human transferrin, IgG, or albumin. Likewise no fluorescence could be detected when strips containing antibodies against human IgG were incubated in solutions of fluorescein-labeled anti-transferrin or anti-albumin antibodies, or when strips with adsorbed antibodies against transferrin were treated with fluorescein-labeled antibodies against IgG or albumin.

If human serum enriched with pure transferrin was allowed to migrate in a strip containing adsorbed antibodies against transferrin the fluorescent area produced by fluorescein-labeled antibodies against human transferrin increased in size compared to the area obtained when the native human serum was allowed to migrate in an identical strip. The recovery of the added transferrin was approximately 100%. Enrichment of the same human serum with human albumin or IgG did not result in any change in the size of the fluorescent area.

*Sensitivity.* When the antibody activity of the immunoglobulin fraction adsorbed to the strip was decreased the fluorescent area produced by



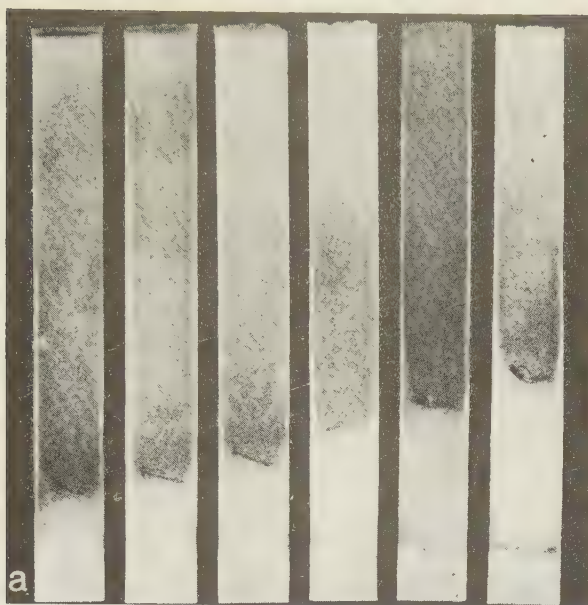


FIG. 1A. Strips with antibodies against human transferrin adsorbed. Serial dilutions of a standard serum were allowed to migrate in the strips and the transferrin-covered areas were determined by fluorescein-labeled antibodies against transferrin. The concentrations of transferrin in the different dilutions were 0.13, 0.16, 0.21, 0.31, 0.42, and 0.63 mg/ml.

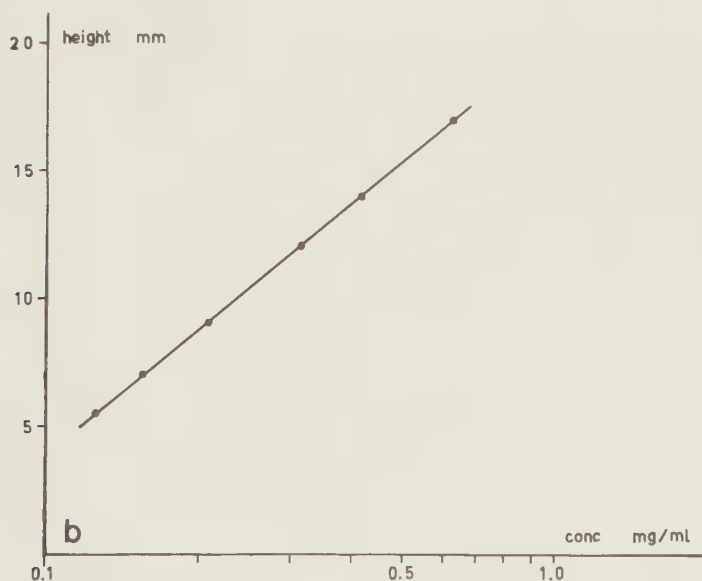


FIG. 1B. Standard curve relating the heights of the fluorescent areas of the strips in Fig. 1A and the antigen concentration of the standard serum dilutions. The heights of the strip portions submerged in the serum dilutions were subtracted from the total heights of the fluorescent areas.

migration of one and the same serum sample increased. But concomitantly with the increase in fluorescent area the intensity of the fluorescence decreased until it was impossible to recognize the boundaries of the fluorescent area. The dose-response curve was linear even for low antibody activities of the immunoglobulin fraction adsorbed to the strip when used for determination of low antigen concentrations. With the detection system described in this work the lowest transferrin and IgG concentrations that could be clearly differentiated from zero were about 40  $\mu\text{g/ml}$ .

*Quantitative comparison with single radial immunodiffusion.* The transferrin concentration in 29 serum samples was determined by immunocapillary migration and by single radial immunodiffusion. A correlation coefficient of 0.91 was obtained when the results of the methods were compared (Fig. 2). The standard curves in the procedures were calculated from dilutions of a human serum with known transferrin concentration.

*Precision.* As determined from duplicate determinations the standard deviation was found to be 11% of the mean for both high-range and low-range concentrations. When a sample series having various antigen concentrations was analyzed repeatedly on 5 successive days the precision was 14% for both high- and low-range concentrations.

*Antiserum consumption.* Calculated from the decrease in immunoglobulin concentration obtained on incubation of the porous material in the antibody solution as determined by absorption measurements at 280 nm, the immunoglobulin amount consumed in the preparation of one strip was found to be about 1 mg. The antibody concentration in the remaining solution was sufficient for preparation of additional strips.

*Storage and stability.* Newly prepared strips and strips stored dry in room temperature in a dark place for 1, 2, and 3 weeks and 1, 3, and 6 months gave results with a coefficient of variation of 14% when tested on six samples of various antigen concentrations stored at  $-24^{\circ}\text{C}$ .

## DISCUSSION

When an antigen solution is allowed to migrate by capillary force in a porous material to which antibodies are attached, the migration of the antigen solute will be specifically delayed in comparison to other molecules of the antigen solution and the relative delay will increase as the antigen concentration decreases. These phenomena form the basis for quantitation by immunocapillary migration.

The specificity, sensitivity, and precision of the procedure will, aside from the properties of the antibodies, be determined by the properties of the porous material, the method used to attach antibodies to the material, and the system chosen to expose the area covered by antigen after migration of the antigen solution.

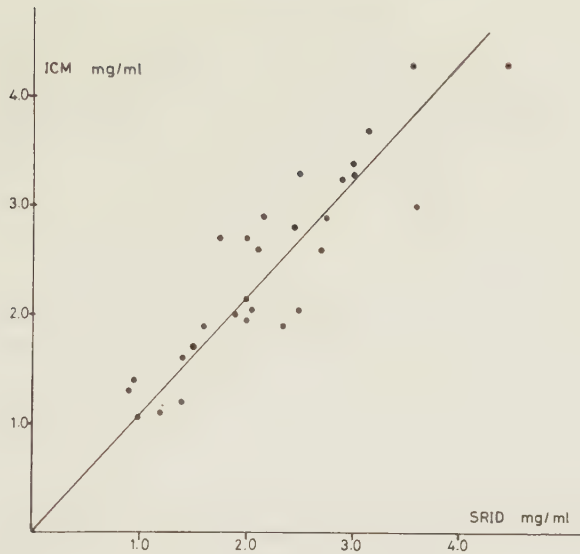


FIG. 2. Relation between the determinations of the transferrin concentration of 29 sera by single radial immunodiffusion (SRID) and by immunocapillarymigration (ICM). The equation  $y = 1.08x$ , where  $y$  is the estimation by ICM, gives the mathematical relation between the estimations as calculated by the method of least squares with zero point statistically weighted.

A porous material ideal for use in immunocapillarymigration should be easy to attach antibodies to and have a structure which promotes rapid and uniform capillarymigration of protein solutions. It should show no nonspecific binding of proteins and be tough enough to allow handling without special precautions. In this study we have used a material composed of polyvinylchloride and a filler, silica gel, which fulfills some of these criteria. Antibodies were attached to the material by simple adsorption and the treated material could be handled without special precautions, showed a rapid uniform capillarymigration of protein solutions and little nonspecific protein binding. Porous materials of cellulose and cellulose acetate were also tested for use in immunocapillarymigration but were found to have several drawbacks. Antibodies could not be adsorbed to these materials. Covalent linkage by comparatively sophisticated procedures involving reagents such as cyanogen bromide or glutardialdehyde was required. The treated materials also demonstrated considerable nonspecific protein binding and were very fragile.

Antibodies could easily be attached to the polyvinylchloride (PVC) material used in this work by a simple adsorption procedure. It is well known that hydrophobic materials like polyvinylchloride adsorb proteins from water solutions (15). The binding of antibodies to the PVC material seems to be comparatively strong since neither prolonged washing with neutral buffers containing 2 M urea nor extensive washing with acid or alkaline buffers could elute the antibodies. The antibodies were adsorbed

to the PVC material from a solution of immunoglobulins containing a high concentration of non-antibody protein molecules. The non-antibody protein serves the important function of saturating the protein binding structures of the PVC material so that the treated material will not show a nonspecific protein binding. Therefore when the amount of antibodies adsorbed to the PVC material is to be varied it is the antibody concentration, not the total concentration of protein, in the incubation medium that should be varied.

We performed some experiments to determine the relation between the amount of antibodies adsorbed to the PVC material and the size of the antigen-covered area produced by migration of an antigen solution in the material. There seemed to be an inverse proportionality between the adsorbed antibody amount and the size of the antigen-covered area, but further experiments are needed to establish this relation for a broad spectrum of antigen concentrations.

In this study a short incubation of the washed PVC material in a solution of fluorescein-labeled antibody was used to expose the antigen-covered area of the material. Several other ways of demonstrating this area are conceivable. The sensitivity of the quantitative procedure may perhaps be increased by the use of antibodies labeled with, e.g., radioactive isotopes or enzymes. If antibodies labeled with colored particles or cells are used, the inspection of the material can perhaps take place in visual light obviating the use of ultraviolet illumination. If trace amounts of antigen labeled in one of the above-mentioned ways are added to the antigen solutions to be analyzed, no incubation in an antibody solution is needed to visualize the antigen-covered area after the capillarymigration.

The antibody-containing material could be used for quantitative purposes without impairment after having been stored for 0.5 years at room temperature demonstrating the stability of antibodies when adsorbed to the porous PVC material. This is in agreement with the common observation that proteins coupled to insoluble-support media often are much more stable than proteins in solution.

Immunocapillarymigration performed as described in this work compares favorably with most other methods for immunochemical quantitation concerning rapidity, simplicity, and stability of reagents. It compares less favorably concerning sensitivity and precision at the present stage but readily allows quantitation of all major plasma proteins. It seems possible to develop immunocapillarymigration into a very rapid, simple, and sensitive quantitative method for general use.

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IMMUNOCAPILLARYMIGRATION WITH ENZYME-LABELLED ANTIBODIES:  
RAPID QUANTITATION OF C-REACTIVE PROTEIN IN HUMAN PLASMA

by

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## ABSTRACT

Various procedures to improve the sensitivity and precision of antigen quantitation by immunocapillarymigration were investigated. The best results were obtained when using porous strips of cellulose acetate with covalently attached antibodies and when enzyme-labelled antibodies were used to expose the antigen-covered areas of the strips. Such a system had a sensitivity of 0.15 mg/l and a precision of 7 %. It allowed a rapid quantitation of human C-reactive protein without the use of laboratory instrumentation.

## INTRODUCTION

We have recently presented a new method for immunochemical quantitation called immunocapillarymigration (1,2). The method is fast and no laboratory instrumentation is required. It is based upon capillarymigration of antigen solutions in immuno-sorbent strips. The antigen-covered area obtained when the migrating antigen binds to antibodies of the strip was exposed by use of fluorescein-labelled antibodies. The sensitivity of the method was not better than 40 mg/l, however, and this prompted studies of how to modify the procedure to allow determinations of clinically important proteins present in low concentrations such as C-reactive protein (CRP). In this work we show that the sensitivity of immunocapillarymigration can be improved to about 0.15 mg/l by use of enzyme-labelled antibodies. Thus, the procedure becomes suitable for routine determinations of C-reactive protein. It is also demonstrated that the modified method has a considerably better precision than the original method.

## MATERIALS

Cellulose acetate sheets were purchased from Sartorius, Göttingen, W.Germany; agarose from Marine Colloides Inc., Rockland, Maine, USA; human transferrin from Kabi AB, Stockholm, Sweden; horse-radish peroxidase type VI and 3-amino-9-ethylcarbazole from Sigma Chemicals Co., St. Louis, Mo., USA; and Sephacryl S-200, Protein A-Sepharose and DEAE-Sephadex A 50 from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of reagent grade.

Monospecific rabbit antisera against human C-reactive protein, serum from non-immunized rabbits, antialbumin-Sepharose and antihemopexin-Sepharose were prepared in the laboratory.

## METHODS

Purification of rabbit IgG. Rabbit serum from non-immunized rabbit and anti-human C-reactive protein antiserum were dialyzed against 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 0.5 mol/l NaCl. The dialyzed sera were applied to a column of Protein A-Sepharose equilibrated with the same buffer. The IgG fraction was eluted from the column with 0.1 mol/l glycine-HCl buffer pH 2.2, containing 0.5 mol/l NaCl. The eluate was concentrated and dialyzed against 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 8 mmol/l sodium azide. IgG concentrations were determined spectrophotometrically using  $A_{280}^{1\%} = 14$  (3).

Purification of human transferrin to be used in recovery experiments. The transferrin preparation was further purified by affinity chromatography on antialbumin-Sepharose and antihemopexin-Sepharose before use, since it contained trace amounts of albumin and hemopexin. The transferrin concentration was determined spectrophotometrically using  $A_{280}^{1\%} = 14.0$  (4).

Preparation of horse-radish peroxidase-labelled antibodies. IgG from rabbit antiserum against C-reactive protein and IgG from goat antiserum against transferrin were conjugated to horse-radish peroxidase according to Wilson et al. (5). The peroxidase-IgG conjugates were purified by chromatography on Sephacryl S-200 in 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 0.1 mol/l NaCl. The absorbance at 280 and 403 nm was determined for all protein-containing fractions and those with a quotient of  $A_{403}/A_{280}$  between 0.3 and 0.7 were combined. These combined fractions contained conjugates with 1-2 peroxidase molecules/antibody molecule (5).

Substrate solution for peroxidase. 3-amino-9-ethylcarbazole was used as substrate for peroxidase (6). Twenty mg 3-amino-9-ethylcarbazole were dissolved in 2.5 ml dimethylformamide. The volume was made up to 50 ml with 0.05 mol/l sodium acetate buffer, pH 5.0. This solution could be stored in the dark at room temperature for a week. Just before use 25  $\mu$ l 30% (v/v) hydrogen peroxide was added. The solution was distributed in 5 ml test-tubes and used as described below. The solution in each test-tube could be used repeatedly until the solution became strongly



coloured.

Covalent coupling of antibodies to cellulose acetate sheets.

Eight cellulose acetate sheets, 150 x 7.5 cm were activated by incubation for 2½ min in a solution containing 1 g CNBr dissolved in 15 ml N-methyl-2-pyrrolidon made up to 100 ml with distilled water (7), followed by 5 min incubation in 2 mol/l Na<sub>2</sub>CO<sub>3</sub>. The sheets were immediately washed in 250 ml 0.1 mol/l NaHCO<sub>3</sub> containing 10 % N-methyl-2-pyrrolidon followed by 750 ml 0.1 mol/l NaHCO<sub>3</sub>. They were then immediately incubated in a solution of 1 mg rabbit IgG against C-reactive protein or goat IgG against transferrin per ml 0.2 mol/l NaHCO<sub>3</sub> (the coupling solution) for 18 h at 4°C. After the incubation the sheets were washed with 0.1 mol/l NaHCO<sub>3</sub> followed by incubation for 2 h in 1 mol/l ethanolamine, pH 8.0. They were then washed with the following solutions: 0.1 mol/l NaHCO<sub>3</sub>, 2 mol/l urea, 0.1 mol/l sodium acetate buffer, pH 4.5 and 0.05 mol/l Tris-HCl buffer, pH 7.4. The sheets were finally dried between filter papers and cut into strips measuring 5 x 70 mm. A mark was made 60 mm from the end of each strip.

Immunocapillarymigration. Plasma or serum samples were diluted with 0.05 mol/l Tris-HCl buffer, pH 7.4. The capillarymigration of samples in the strips was allowed to proceed as described in detail earlier (2) (Fig. 1). Briefly, the unmarked end of a strip was placed in a small cup containing 300 µl of sample. After capillarymigration of the sample to the mark at 60 mm the strip was washed for 1 min in 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 0.02 % (v/v) Tween 20. The antigen-covered area was then exposed in two steps. First, the strip was incubated for 3 min in a solution of horse-radish peroxidase-labelled antibodies. Excess peroxidase-labelled antibodies were washed off by incubation for approximately 5 min in the Tris-Tween buffer described above. The strip was then incubated in a test-tube containing peroxidase-substrate solution. When a dense coloured area had developed the strip was removed from the solution. The height of the coloured area was measured with a ruler and the corresponding antigen concentration was determined from a standard curve.

Electroimmunoassay. The procedure of Laurell (8) was followed. A

0.075 mol/l sodium barbital buffer, pH 8.6, containing 2 mmol/l EDTA was used. The electrophoresis was continued for 18 h at 10 V/cm.

## RESULTS

When the antigen-covered areas obtained after migration of samples in antibody-containing strips were exposed by peroxidase-labelled antibodies and the substrate 3-amino-9-ethylcarbazole, heavily stained reddish-brown areas were observed within 2½ min (Fig. 2).

Sensitivity. By mixing a coupling solution of rabbit IgG against C-reactive protein with a solution of rabbit IgG from non-immunized rabbits with the same IgG concentration, the concentration range for which the strips could be used was decreased. In this way it was possible to prepare strips allowing determinations of normal concentrations (<5 mg/l) of C-reactive protein. At a lower antigen-level a lower amount of enzyme-labelled antibodies were bound to the antigen-covered area and hence there was a slower rate of colour production. This could be compensated for by longer incubation times with the substrate. Since a small amount of non-specifically bound enzyme-labelled antibodies always remains in the strips a prolonged incubation in substrate solution also increases the background colour. The lowest concentration that could be clearly differentiated from zero with this developing system was 0.15 mg/l.

Recovery. If human serum was diluted and transferrin added before migration in a strip containing goat IgG against transferrin there was an increase in the stained area compared to that obtained with the dilution without extra added transferrin. The recovery of the added transferrin was 97-105 % when measurements were carried out at 2 transferrin concentrations.

Precision. The standard deviation determined from duplicate

determinations of C-reactive protein was 7 % of the mean for both high and low range values.

Analysis time. The time to complete an assay was approximately 60 min and around 30 samples could be handled simultaneously.

Quantitative comparison with electroimmunoassay. The concentration of C-reactive protein in 40 human plasma samples was determined with immunocapillarymigration and electroimmunoassay using the same rabbit immune IgG. The standard curves were calculated from serial dilutions of a plasma sample with a known concentration of C-reactive protein. Usually samples were diluted with 10 times for electroimmunoassay and 50 times for immunocapillarymigration. If the concentration was too high or too low the samples had to be reanalyzed by both methods. The sensitivity of the electroimmunoassay used was 2.5 mg/l, while it was 0.75 mg/l for the immunocapillarymigration system. Therefore when the two methods were compared all values below 2.5 mg/l for both methods were registered as nil. The comparison gave a correlation coefficient of 0.95 (Fig. 3).

Stability. The antibody-containing strips could be stored dry at room temperature in the dark. A comparison of newly prepared strips and strips from the same preparation that had been stored for 9 months gave results with a coefficient of variation of 8 % when tested on four plasma samples at various antigen concentrations. The plasma had been stored at -24°C.

## DISCUSSION

In the present work we demonstrate that by using enzyme-labelled, instead of fluorescein-labelled, antibodies to expose the antigen-covered areas an improvement of the sensitivity to about 0.15 mg/l was obtained. It also gave an improvement in precision from 11 % to 7 %. This is probably due to the fact that more accurate measurements of the areas could be made in

visible light than in UV-light.

There are two requirements in the choice of enzyme for labelling antibodies used in immunocapillarymigration. The enzyme should easily conjugate with antibodies yielding well defined conjugates and it should catalyze a reaction which gives a coloured insoluble product precipitating at the site of the enzyme in the strip. Horse-radish peroxidase was found to fulfil these requirements. This enzyme can be coupled to amino groups in the antibody molecule via its two available lysine residues by use of the bifunctional reagent glutardialdehyde as described by Avrameas et al (9). Alternatively it can be coupled through its carbohydrate side chain by use of periodate oxidation (5). In our preliminary investigation with enzyme-labelled antibodies (10), the two-step glutardialdehyde method was used for conjugation. However, the method was unsatisfactory for two reasons. First, the coupling yield was low calculated from the amount of conjugates obtained compared with the amount of enzyme that had to be used (11). Second, the conjugates obtained had a rather low enzyme activity and consequently, a comparatively long incubation time with the substrate was required to get enough staining (10). In this work we have used enzyme conjugates obtained by the periodate method. These conjugates have a comparatively high enzyme activity. This was demonstrated by the shorter incubation time required to get a heavily stained area. Although the reaction product is almost completely insoluble there is always a small soluble fraction which diffuses from the reaction site in the strip causing a blurred boundary.

In the original work on immunocapillarymigration we used strips of polyvinyl chloride with silica gel as a filler as the porous support. This material was unsuitable to use in this case, since enzyme-labelled antibodies were non-specifically bound to this material. A search for other porous materials with a low non-specific binding of enzyme-labelled antibodies revealed that cellulose acetate is a suitable material. Antibodies were covalently attached to the material by the CNBr-method (12). However, the described procedure had to be changed since stir-

ring of the reaction solution caused rupture of the cellulose acetate sheets. Without stirring a very poor activation was obtained due to the fact that the decomposition rate of CNBr exceeded its rate of diffusion into the sheets. This obstacle was overcome by performing the activation in two steps. The sheets were first incubated in an unbuffered solution of CNBr of neutral or low pH to allow a diffusion of CNBr into the sheets. This was followed by an incubation in a strong  $\text{Na}_2\text{CO}_3$ -solution of high pH to start the reaction. The  $\text{Na}_2\text{CO}_3$ -solution was strong enough to keep the pH around 10.5 throughout the reaction and therefore titration with NaOH could be omitted.

To obtain a rapid method it is important that the washing step between the incubation in labelled antibodies and the substrate reaction is efficient. Different buffers with varying ionic strength and pH were tested. It was found that high ionic strength influenced the following substrate reaction. The activity of the enzyme was decreased and a brownish-red product was produced. Since the substrate reaction is optimal at pH 5.0, washing buffers at this pH were tested, but was found to be less efficient than Tris-HCl buffer, pH 7.4. The overall best result was obtained when double distilled water was used in both washing-buffer and substrate solution, since the substrate reaction can be influenced by small amounts of metal-ions.

After the migration the antigen-covered area contains both antigen molecules bound to antibodies in the material and free antigen molecules (13). Thus, the short washing step before incubation of the strips with labelled antibodies is crucial for the success of the procedure. If this step is omitted the free antigen will be washed off in the solution of enzyme-labelled antibodies and react with the antibodies, causing rapid depletion of the antibodies. The antigen-antibody conjugates produced may react with antibodies in the strip giving rise to a high background colour.

The concentration of C-reactive protein in human plasma or serum



is a very sensitive indicator of inflammatory processes and several methods have been developed for quantitation of the protein. Radioelectroimmunoassay (14) is the most sensitive of these techniques, but more than one day is required for analysis and the method is therefore too slow for routine clinical use. Conventional electroimmunoassay (15) is more rapid but nevertheless an analysis requires several hours. Laboratory instrumentation and technicians are also needed for this method. Latex-agglutination tests, which are commercially available, are rapid and simple. These tests are only semi-quantitative, however, and may give false negative results for very high concentrations of C-reactive protein. Immunocapillarymigration is characterized by its rapidity and that it does not need any instrumentation for its performance. We think therefore that this method will be a powerful tool in the quantitation of C-reactive protein and other plasma proteins in acute situations and/or when no laboratory instrumentation is available. We also believe that the sensitivity of immunocapillarymigration can be improved further by use of radioactive ligands as indicated in a brief communication concerning the quantitation of insulin by a similar procedure (16).

#### ACKNOWLEDGEMENT

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## LEGENDS TO FIGURES

Figure 1. A scheme of the immunocapillarymigration procedure.

Figure 2. Stained immunocapillarymigration strips. Serial dilutions of a human plasma were allowed to migrate in strips with covalently bound antibodies against C-reactive protein. After migration the antigen-covered areas of the strips were exposed by successive incubations in solutions of peroxidase-labelled antibodies against C-reactive protein and the peroxidase substrate 3-amino-9-ethylcarbazole. The C-reactive protein concentrations were 4.8, 2.4, 1.2, 0.60, 0.30, and 0.15 mg/l.

Figure 3. A comparison between determinations of C-reactive protein concentrations in 40 human samples by electroimmunoassay (EIA) and immunocapillarymigration (ICM). The equation  $Y = 0.98X + 2.28$ , where Y is the estimation by ICM, gives the mathematical relation between the estimations as calculated by the method of least squares.

Fig. 1.

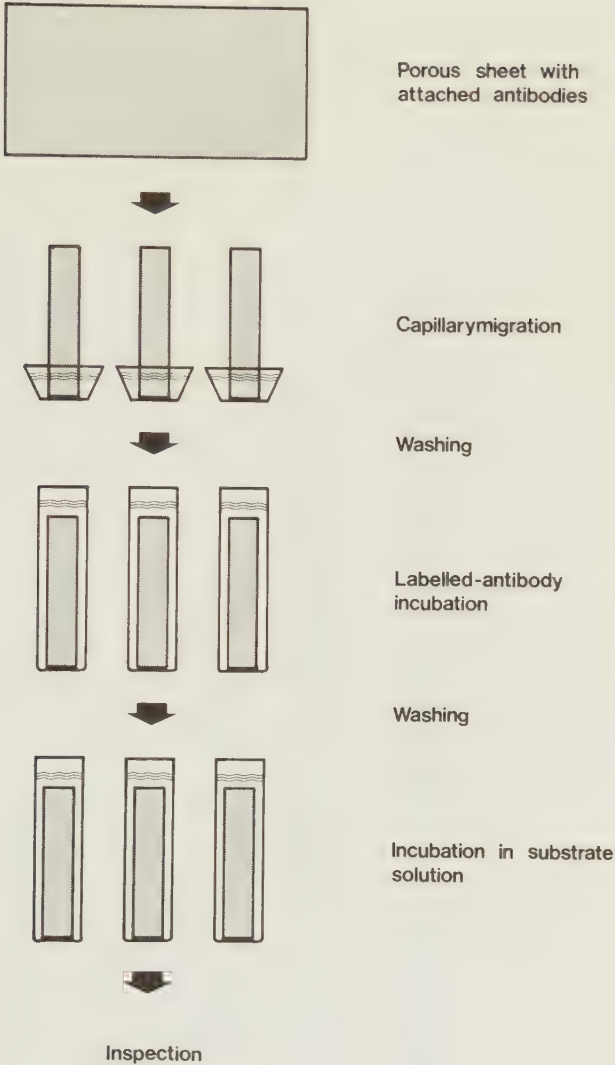


Fig. 2.

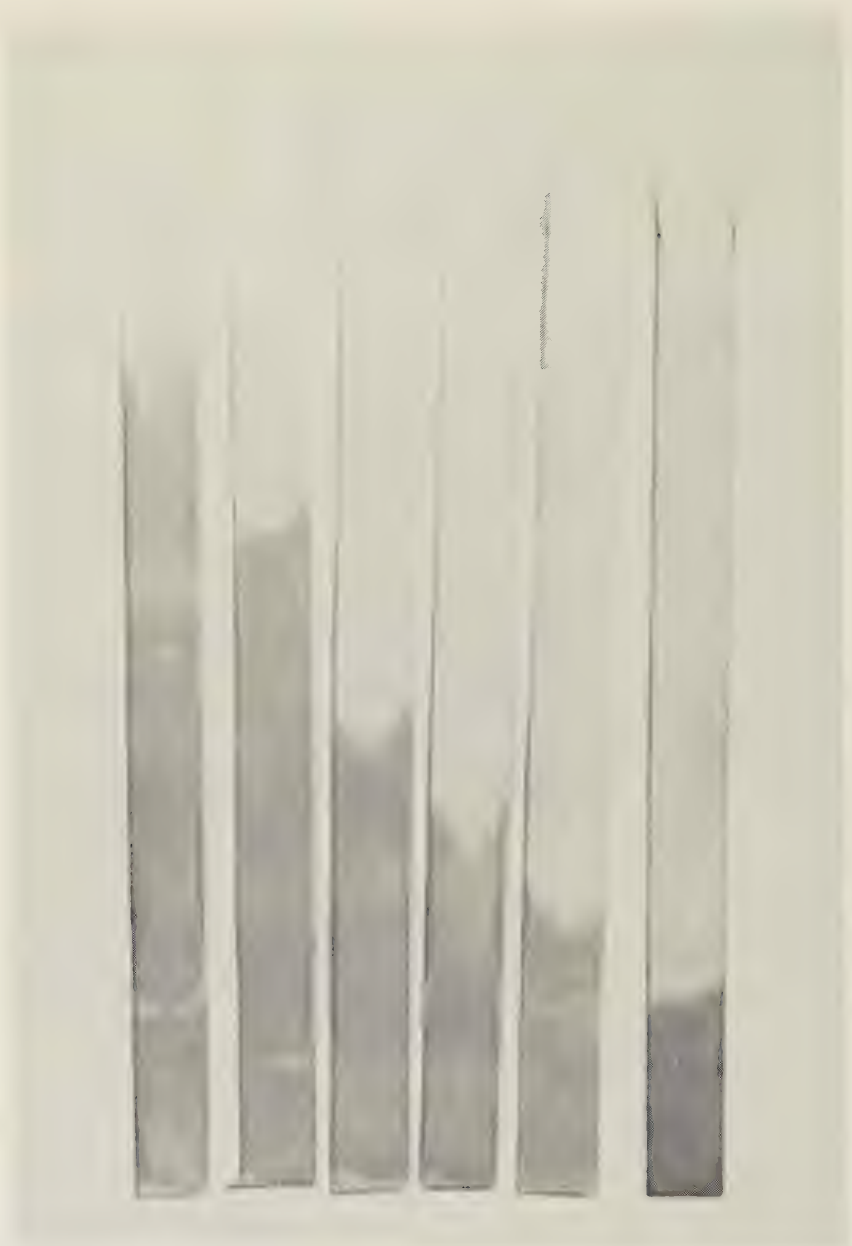
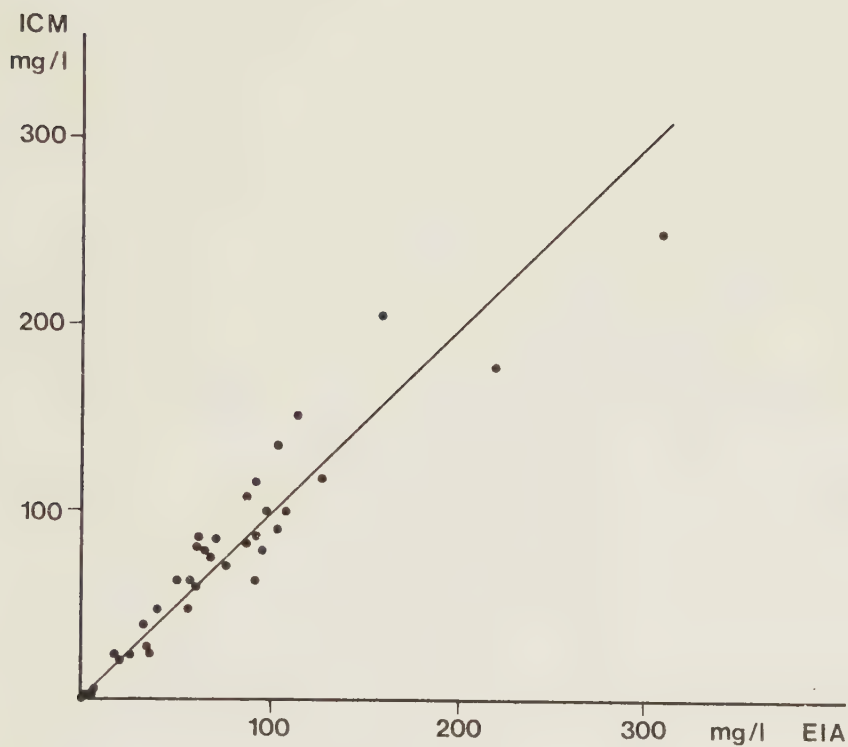


Fig. 3.











STUDIES ON THE MECHANISM OF IMMUNOCAPILLARYMIGRATION  
AND INFLUENCING FACTORS

by

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## ABSTRACT

The interaction between migrating transferrin and coupled anti-transferrin antibodies in immunocapillarymigration strips was studied with a two-step migration procedure. The binding between antigen and antibody during the capillarymigration was rather stable under the experimental conditions. About 20 % of the bound transferrin could be replaced by new transferrin, but the bound transferrin could not be washed off the strip. A study of how the amount of coupled antibodies in the supporting material influences the concentration of antigen that could be analyzed showed a proportionality between the two parameters. The protein concentration of the sample solution had no effect on the quantitative determination, unless the migration rate of the sample was affected. When undiluted serum was the sample the migration of antigen was retarded in comparison to diluted serum or when the antigen was contained in buffer. As a consequence the estimated concentration of antigen in undiluted serum was too low.

## INTRODUCTION

Immunocapillarymigration is a quantitative method based upon the migration of antigen in an antibody-containing material (1,2,3). During the capillarymigration the antigen interacts specifically with antibodies in the material and is retarded compared to other constituents in the migration solution. An antigen-covered area is thus obtained. The height of this area increases as the concentration of antigen in the sample increases.

This work was undertaken to study how the antigen interacts with the attached antibodies in the material, and how changes in the amount of attached antibodies and other factors influence the relative delay of the antigen.

## MATERIALS

Celluloseacetate sheets were purchased from Sartorius, Göttingen, W. Germany; horse-radish peroxidase type VI and 3-amino-9-ethylcarbazole from Sigma Chemical Co., St. Louis, Mo., USA; human transferrin from Kabi AB, Stockholm, Sweden; DEAE-Sephadex A50 from Pharmacia Fine Chemicals, Uppsala Sweden. All other chemicals were of reagent grade.

Monospecific goat antiserum against human transferrin, non-immune goat serum, non-immune rabbit serum, anti-albumin-Sepharose, anti-hemopexin-Sepharose and rabbit antiserum against total human serum were prepared in the laboratory.

## METHODS

Preparation of IgG from goat serum: Goat serum was dialyzed against 0.1 mol/l Tris-HCl buffer, pH 7.2, and applied on a column of DEAE-Sephadex A50 equilibrated with the same buffer. The run-through fraction contained the IgG of the serum and was collected and dialyzed against distilled water and lyophilized.

Purification of human transferrin: The transferrin preparation was purified by affinity chromatography on anti-albumin-Sepharose and anti-hemopexin-Sepharose before use, since it contained trace amounts of albumin and hemopexin when analysed by immunoelectrophoresis (4) against antiserum against total human serum.

Iodination procedure: The purified transferrin was labelled by iodination with I-125 according to Thorell and Johansson (5) utilizing a lactoperoxidase solution.

Preparation of fluorescein-labelled antibodies: IgG from goat serum against transferrin was labelled with fluorescein isothiocyanate as described earlier (2).

Preparation of horse-radish peroxidase labelled antibodies: IgG from goat antiserum against transferrin was conjugated to horse-radish peroxidase according to Wilson and Nakane (6).

Substrate solution for peroxidase: 3-amino-9-ethylcarbazole was used as substrate for peroxidase (7). Twenty mg 3-amino-9-ethylcarbazole was dissolved in 2.5 ml dimethylformamide. The volume was made up to 50 ml with 0.05 mol/l sodium acetate buffer, pH 5.0, Just before use 25  $\mu$ l 30 % (v/v) hydrogen peroxide was added. The solution was distributed in 10 ml test-tubes and used as described below.

Covalent coupling of antibodies to cellulose acetate: Eight cellulose acetate sheets, 150 x 7.5 cm, were activated by incubation for 2½ min in a solution containing 1 g CNBr dissolved in 15 ml N-methyl-2-pyrrolidone made up to 100 ml with distilled water, followed by 5 min incubation in 2 mol/l Na<sub>2</sub>CO<sub>3</sub>. After washing with 0.1 mol/l NaHCO<sub>3</sub> the sheets were immediately incubated in a solution of 1 mg goat IgG against human transferrin per ml 0.2 mol/l NaHCO<sub>3</sub> (the coupling solution) for 18 h at 4°C. After the incubation the sheets were washed with 0.1 mol/l NaHCO<sub>3</sub>, incubated for 2 h in 1 mol/l ethanolamine, pH 8.0, and successively washed with the following solutions: 0.1 mol/l NaHCO<sub>3</sub>, 2 mol/l urea, 0.1 mol/l sodium acetate buffer, pH 4.5 and 0.05 mol/l Tris-HCl buffer, pH 7.4. The sheets were finally dried between filter papers and cut into strips measuring 5 x 70 mm. A mark was made 60 mm from the end of each strip.



Immunocapillarymigration: The capillarymigration of samples was performed as described in detail earlier (2). Briefly, the unmarked ends of the strips were placed in small cups containing 300  $\mu$ l of the samples. The samples were allowed to migrate by capillary force to the mark made at the top of each strip. After migration the strips were washed for 1 min in 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 0.02 % Tween 20. The antigen-covered areas were then exposed by incubating the strips for 3 min in a solution of antibodies labelled with horse-radish peroxidase. Excess labelled antibodies were washed off the strips by incubation for 5 min in the Tris-Tween buffer used above. The strips were then incubated in test-tubes containing substrate solution for peroxidase. Densely coloured areas developed within 2½ min and the strips were removed from the solution. The heights of these areas were measured and the corresponding antigen concentrations were obtained from standard curves.

When the antigen covered area was detected with fluorescein-labelled antibodies, the strip was incubated for 3 min in a solution of these antibodies. It was then washed in water and inspected at 254 nm.

Two-step migration procedure: An additional mark was made at the height of 30 mm on each strip. The capillarymigration in these strips was then performed as follows. A solution of I-125 labelled transferrin was allowed to migrate to the first mark, the strip was removed from the solution and excess solution at the bottom of the strip was blotted off. The strip was immediately transferred to a cup containing I-125-transferrin, unlabelled transferrin or 0.05 mol/l Tris-HCl buffer, pH 7.4, and the migration was allowed to continue to the second mark on the strip. The strip was dried and cut into fourteen 5 mm pieces which were counted in a gamma counter.

## RESULTS AND DISCUSSION

Capillarymigration. To investigate the mechanism operative during capillarymigration a two-step migration procedure was used. In the first step labelled transferrin migrated to a

height of 30 mm in the immunosorbent strip. The strip was transferred to a solution of unlabelled transferrin or a buffer solution and the migration proceeded. The distribution of labelled transferrin obtained in the strip was compared to the distribution obtained after the first migration step and that obtained with labelled transferrin in both steps. It was thus possible to study how the interaction between labelled transferrin and antibodies obtained in the first step was influenced by the solutions migrating in the second step. The results obtained are shown in figure 1 together with results obtained in strips containing non-immune goat IgG.

When labelled transferrin migrated in a strip containing non-immune IgG, the transferrin became evenly distributed in the strip (Fig. 1A). In such a strip there was no retardation of transferrin (Fig. 1B). Thus, the retardation obtained in a strip containing antitransferrin is due to specific interactions between transferrin and attached antibodies, and not to unspecific interactions with cellulose acetate.

In immunocapillarymigration the protein to be quantified, the antigen, is a constituent of the migrating solution. Consequently, new antigen is entering the strip during the entire capillarymigration. When antigen migrates in an antibody-containing strip antigen-antibody interactions will produce an antigen-covered area. When this area has started to form there will be no more binding of antigen when all antibodies in the area is occupied. The migrating solution in this area will then have the same concentration of free antigen as is found in the sample or in a strip containing non-immune IgG. Thus, the antigen-covered area obtained in the first step (Fig. 1C) will contain both free and bound antigen. When buffer migrates in the second step this free antigen will continue to migrate until all of it is bound to antibodies. A decrease of the antigen concentration in the covered area combined with an increase in area will be obtained (Fig. 1D). A comparison of the decrease in concentration of labelled transferrin obtained during the second step (2500 - 1500 cpm) and the concentration of free transferrin

in a strip containing non-immune IgG (950 cpm; Fig. 1A) shows that the decrease in concentration is only due to loss of free transferrin. The results obtained with buffer in the second step (Fig. 1D) was compared with results obtained when this step was replaced by washing. The decrease in concentration of labelled transferrin was the same in both strips, but in the washed strip the covered area was not increased. These results show that a stable transferrin-antitransferrin interaction is obtained during capillary migration.

When transferrin is migrating in both steps the transferrin-covered area will be exposed to free transferrin during the entire migration. An exchange of transferrin in the area may then occur. To determine if free transferrin replaces already bound transferrin, unlabelled-transferrin was migrated in the second step (Fig. 1E). The results show a greater decrease in concentration of labelled-transferrin than that obtained with buffer migrating in the second step. A comparison of the concentrations (1500 cpm compared to 1200 cpm) suggests that approximately 20 % of the bound transferrin is exchanged.

An even distribution of radioactivity was seen when unlabelled-transferrin migrated in the first step and labelled-transferrin in the second (Fig. 1F). This may at first appear to disagree with the results discussed above. However, since the antigen-covered area is composed of bound and free antigen a large fraction of the radioactivity constitutes free antigen. The radioactivity that has bound during the second step can be calculated by subtracting free antigen (950 cpm) from the values obtained.

A slight increase in the concentration of transferrin in the covered area was seen after the second step (Fig. 1C and G). Since the concentration of free antigen is the same, the increase must result from a higher amount of bound antigen. In the strip all attached antibodies will not be equally accessible and the attachment procedure may have affected the antibody activity. Thus, the reactivity towards transferrin will vary. A

binding between transferrin and less reactive antibodies will require a longer contact time. This is obtained during the second step since the rate of migration decreases with the height of migration. Thus, the observed increase in concentration of bound labelled transferrin is due to utilization of a higher amount of attached antibodies.

The effect of the concentration of specific antibodies in the coupling solution. The height of the antigen-covered area that is formed in a strip is dependent on the antigen concentration in the sample and the concentration of specific antibodies in the strip. Since less antigen can be quantified with a lower amount of specific antibodies in the strip (2) it was examined whether a simple relation exists between these parameters. Coupling solutions with decreasing amounts of specific antibodies to transferrin but with a constant amount of IgG were prepared by the addition of non-immune IgG. These were used to prepare strips containing different amounts of specific antibodies. The strips were used for analysis of serial dilutions of human serum. A comparison of the standard curves obtained with strips prepared from undiluted and 2-fold diluted coupling solution was performed (Fig. 2). It shows that antigen-covered areas of the same height are obtained when the sample and the coupling solution are diluted to the same extent. The same relation was found when the coupling solution was diluted over a wider range (table 1). Hence, the amount of specific antibodies in the strip is proportional to the antigen concentration that can be analyzed.

The influence of buffer composition, ionic strength and pH of the sample on capillary migration. It is known from studies of the precipitin reaction between antigen and antibody, that the reaction is influenced by several factors, for instance the composition of the buffer (8) and the protein concentration of the diluents (9). Particularly, the secondary reaction leading to precipitation is influenced. The primary antigen-antibody interaction is known to be influenced only by extreme pH ( $> \text{pH } 9$  and  $< \text{pH } 4$ ) and high salt concentrations ( $> 12\% \text{ (w/v) NaCl}$ ).

Although the primary reaction is the only one that takes place in immunocapillarymigration, the influence of the composition of the buffer, ionic strength and pH of the sample on capillarymigration was tested.

Human serum was diluted with different buffers and the capillarymigration was tested using strips with coupled anti-transferrin. The migration distance for transferrin became longer when samples were diluted with barbital buffer than with Tris or phosphate buffer (table 2), although the transferrin concentration was the same in all samples. Serial dilutions of the serum with 0.05 mol/l Tris-HCl buffer, pH 7.4, or 0.075 mol/l barbital buffer, pH 8.6, also containing 2 mmol/l EDTA, followed by immunocapillarymigration showed that this difference in migration distance occurred over the entire concentration range tested (Fig. 3). The migration distance was not affected by pH and/or ionic strength with other buffers than sodium barbital. The pronounced increase in height obtained with barbital buffer indicates that a less efficient interaction between antigen and antibody is obtained in this case. This is probably due to a binding of barbital ions to transferrin resulting in a masking of antigenic determinants in this molecule. It is, therefore, important that the standard curve is determined using the same buffer as is present in the sample, particularly if this buffer contains ions known to bind to proteins.

The influence of the protein concentration in the sample on capillarymigration. The importance of similar composition of standards and samples is often stressed in the discussion of results obtained with various analytical systems. In constructing the standard curve for an immunocapillarymigration assay, serial dilutions of a standard serum or plasma is used. Hence, the protein concentration of the highest and lowest point in the standard curve will differ substantially. To study if the protein concentration has any effect on the standard curve, human serum was diluted 25-fold with 0.05 mol/l Tris-HCl buffer, pH 7.4, and was divided into two parts. One part was serially diluted with the buffer and the other part was serially diluted



with an already 25-fold diluted rabbit serum. The diluted samples were then analysed by migration in strips containing antitransferrin. Identical standard curves were obtained in both dilution series (Fig.4). Thus, variations in the protein concentration do not influence the standard curves at the protein level tested. It was also examined if samples with the same antigen concentration, but with various amounts of protein, gave the same quantitative result. Such samples were obtained by adding 10  $\mu$ l of a human serum containing the antigen to one ml of serial dilutions (1:1, 1:2, 1:4, 1:8) of rabbit serum. When analyzed on strips containing anti-transferrin the sample with undiluted rabbit serum migrated much slower than did any of the other samples, and the transferrin-covered area obtained was lower. In all samples where the rabbit serum had been diluted the same height and therefore the same transferrin concentration was obtained. Thus, a variation of the protein concentration in the sample does not influence the quantitative result unless the concentration is so high that the migration rate is influenced. For such samples the estimated concentration will be too low.

## CONCLUSION

The fact that the antigen-covered area contains free antigen stresses the importance of a washing step between capillary-migration and the incubation with labelled antibodies. If the strip was incubated directly with labelled-antibodies the free antigen would be washed off in the solution. The antigen would then react with the labelled antibodies and rapidly consume these.

The experiments concerning the replacement of already bound antigen and the inability to wash off bound antigen indicate that a rather stable binding between antigen and antibody is formed during the capillarymigration. This is important, since a less stable binding may cause a release of bound antigen during the incubation with labelled antibodies, and thus reduce the

sensitivity of the method.

The performance of an immunocapillarymigration assay is made simpler by the fact that the antigen concentration that can be analyzed is proportional to the concentration of specific antibodies in the coupling solution used when the strips are prepared. Once a set of strips has been prepared and a standard curve constructed, the result can be used for preparation of new strips in the concentration range wanted. It is important, however, that the IgG concentration in the coupling solutions is the same. When covalent coupling is used it is also important that the CNBr preparation is of the same quality, since variations in the activation of the material influences the amount of coupled antibodies.

#### ACKNOWLEDGEMENT

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TABLE 1

The relation between the dilution of the coupling solution, the dilution of the sample and the height of the coloured area obtained on the strips.

| Dilutions of<br>coupling solution | Sample dilution<br>(2 g transferrin/l) | Height of coloured<br>area (mm) |
|-----------------------------------|----------------------------------------|---------------------------------|
|-----------------------------------|----------------------------------------|---------------------------------|

---

|      |        |      |
|------|--------|------|
| -    | 1:200  | 26   |
| 1:2  | 1:400  | 26.5 |
| 1:4  | 1:800  | 25   |
| 1:10 | 1:2000 | 25.5 |

TABLE 2

The heights of antigen-covered areas produced in strips containing antibodies against transferrin when different buffers were used to dilute a standard serum 1:100. The averages of three runs are presented.

| Buffer | Height of antigen-covered area (mm) |
|--------|-------------------------------------|
|--------|-------------------------------------|

---

|                                                                   |      |
|-------------------------------------------------------------------|------|
| 0.05 mol/l Tris-HCl, pH 7.4                                       | 27   |
| 0.05 mol/l Tris-HCl, pH 8.6                                       | 30   |
| 0.1 mol/l Tris-HCl, pH 8.6                                        | 30   |
| 0.075 mol/l barbital buffer, pH 8.6 +<br>2 mmol/l calcium lactate | 40   |
| 0.075 mol/l barbital buffer, pH 8.6 +<br>2 mmol/l EDTA            | 39   |
| 0.05 mol/l sodium phosphate, pH 5.7                               | 27   |
| 0.05 mol/l sodium phosphate, pH 6.7                               | 25.5 |
| 0.1 mol/l sodium phosphate, pH 6.7                                | 26   |
| 0.01 mol/l sodium phosphate, pH 6.7                               | 25.5 |
| 0.15 mol/l sodium chloride                                        | 26   |

## LEGENDS TO FIGURES

- Figure 1. Strips used in the two-step migration procedure. The radioactivity (cpm) found in each piece of the strips is written beside the strip. All values are given with the background (100 cpm) subtracted. The part of the strips that was inserted in the migrating solution has been marked. Tf = transferrin. A-B: cellulose acetate strips with non-immune goat IgG coupled. C-G: cellulose acetate strips with IgG from goat antiserum against human transferrin coupled.
- Figure 2. Standard curves relating the height of coloured areas obtained and the transferrin concentration of the sample. (■) undiluted coupling solution, (●) coupling solution two-fold diluted with a solution of non-immune goat IgG
- Figure 3. Standard curve relating the height of coloured areas obtained and the transferrin concentration of the sample when 0.05 mol/l Tris-HCl buffer, pH 7.4, (■) and when 0.075 mol/l barbital buffer, pH 8.6, containing 2 mmol/l EDTA (●) were used as diluents.
- Figure 4. Standard curves relating the heights of the coloured areas obtained and the transferrin concentration of the sample when 0.05 mol/l Tris-HCl buffer, pH 7.4 (■) and when rabbit serum diluted 25-fold with Tris-buffer (○) were used for serial dilutions of a serum sample. The serum was pre-diluted 25 fold with Tris-buffer.

Fig. 1.

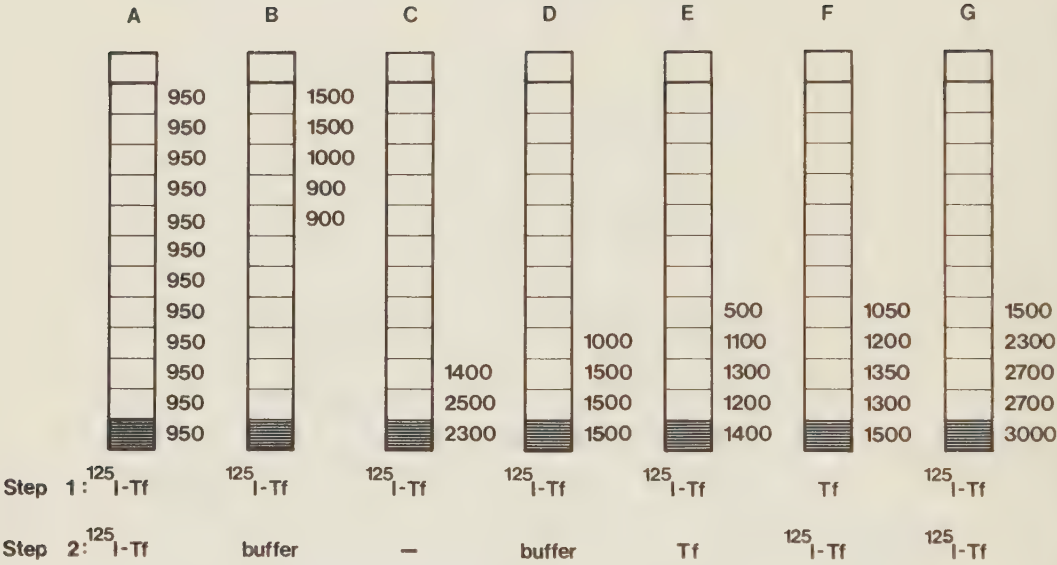


Fig. 2.

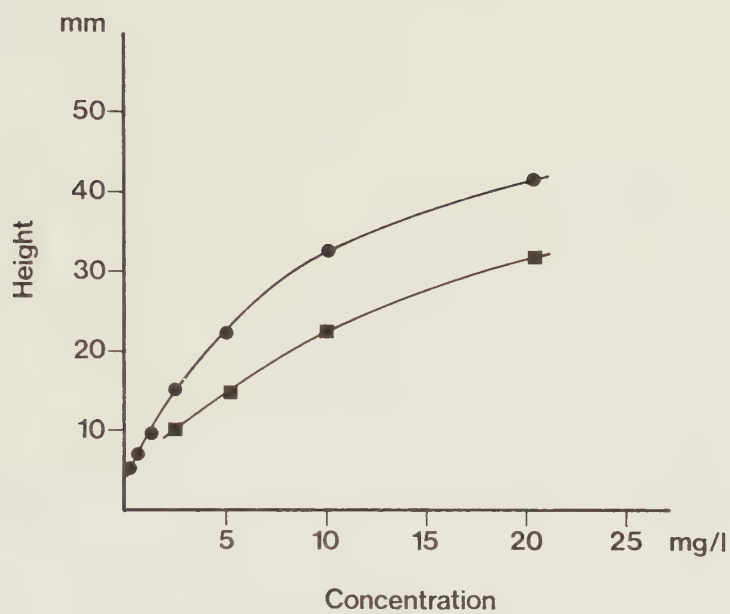




Fig. 3.

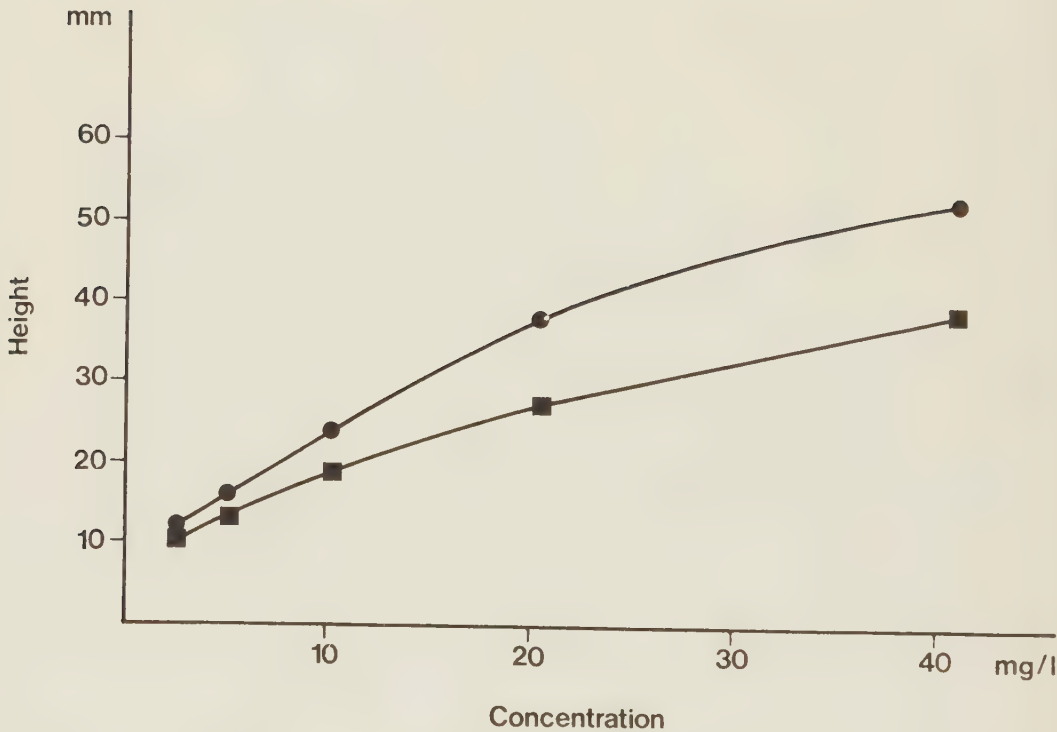
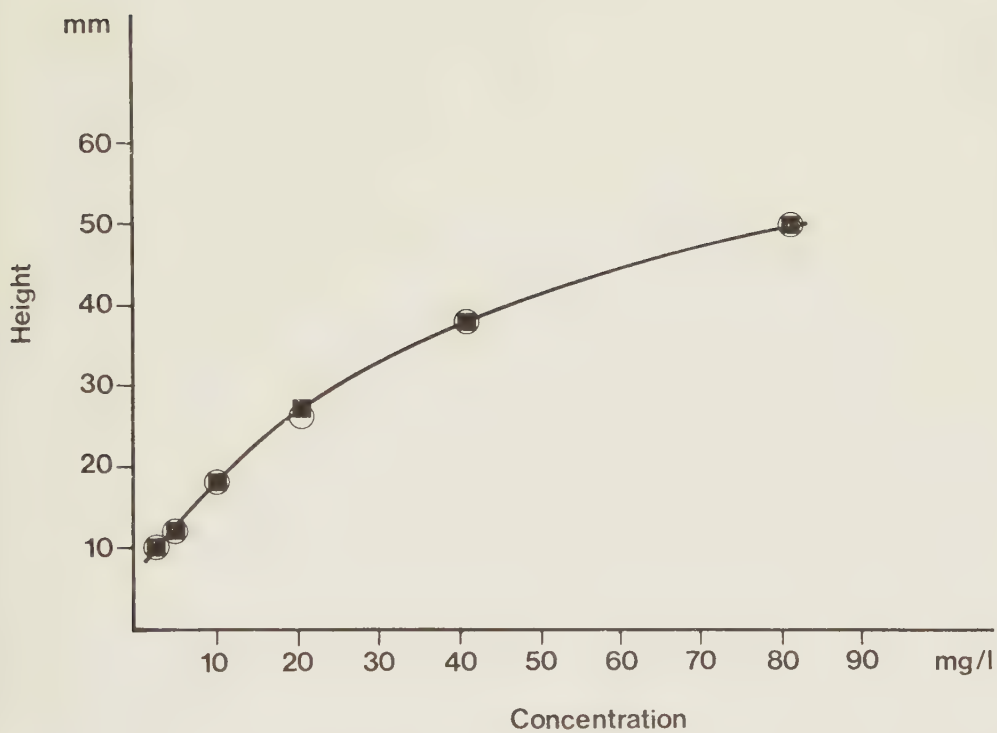


Fig. 4.





V



VERY RAPID QUANTITATION OF C-REACTIVE PROTEIN BY  
IMMUNOCAPILLARYMIGRATION IN CHROMATOGRAPHY PAPER

by

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## ABSTRACT

A modification of immunocapillarymigration is described. By the use of chromatography paper as the porous support for the capillarymigration, C-reactive protein could be quantified within 15 minutes. Concentrations down to 0.3 mg/l of C-reactive protein could be quantified in a plasma sample with a precision of 8 %.



## INTRODUCTION

Immunocapillarymigration was developed as a simple and rapid immunochemical method for quantitation of proteins (1,2). In its original design the method had a relatively low sensitivity and precision compared to methods like single radial immunodiffusion (3) and electroimmunoassay (4) commonly used for quantitation of proteins. A modification of the method has lately been done (5). The fluorescein-labelled antibodies originally used to expose the antigen-covered areas of the strips were replaced by enzyme-labelled antibodies. This increased the sensitivity about 200 times and improved the precision considerably.

The immunocapillarymigration procedure requires 60 min for quantitation of proteins. Most of this time is required for the capillarymigration of the sample in the strip. Since the migration is slow it has to be performed in a humidified chamber to minimize the evaporation from the strip. This work shows that the migration time can be reduced to 5 min when strips of chromatography paper are used, and that the humidified chamber can be obviated.

## MATERIALS

Whatman chromatography paper No. 3 was purchased from Whatman Ltd., Maidstone, England; horse-radish peroxidase type VI and 3-amino-9-ethylcarbazole from Sigma Chemicals Co., St. Louis, Mo., USA; bovine serum albumin from Miles Laboratories Inc., Kankakee, Ill., USA; Protein A-Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were of reagent grade.

Monospecific rabbit antiserum against human C-reactive protein was available at the laboratory.

## METHODS

Preparation of IgG from rabbit serum. The IgG fraction of rabbit serum was isolated by affinity chromatography on Protein A-Sepharose. The IgG fraction was eluted from the column with 0.1 mol/l glycine-HCl buffer, pH 2.2, also containing 0.5 mol/l NaCl. The eluate was concentrated and dialyzed against 0.05

mol/l Tris-HCl buffer, pH 7.4, also containing 8 mmol/l sodium azide.

Preparation of horse-radish peroxidase labelled antibodies. IgG from rabbit antiserum against human C-reactive protein was conjugated to horse-radish peroxidase according to Wilson et al. (6).

Peroxidase-substrate solution. Twenty mg 3-amino-9-ethylcarbazole was dissolved in 2.5 ml dimethylformamide. The volume was made up to 50 ml with 0.05 mol/l sodium acetate buffer, pH 5.0, and 25  $\mu$ l 30 % (v/v) hydrogen peroxide was added. The solution was prepared just before use and was protected from light.

Covalent coupling of antibodies to Whatman chromatography paper No. 3 with CNBr. Four sheets, 70 x 150 mm, were activated by incubation for 2½ min in a solution of 1 g CNBr dissolved in 15 ml N-methyl-2-pyrrolidone made up to 100 ml with distilled water (7), followed by 5 min incubation in 2 mol/l Na<sub>2</sub>CO<sub>3</sub>. The sheets were then washed with 250 ml 0.1 mol/l NaHCO<sub>3</sub> containing 10 % N-methyl-2-pyrrolidone followed by 750 ml 0.1 mol/l NaHCO<sub>3</sub>. They were then immediately incubated in a solution of 1 mg rabbit IgG against C-reactive protein per ml 0.2 mol/l NaHCO<sub>3</sub> (the coupling solution) for 18 h at 4°C. After the incubation the sheets were washed with 0.1 mol/l NaHCO<sub>3</sub> and incubated for 2 h in 1 mol/l ethanolamine, pH 8.0. The sheets were then washed consecutively with the following solutions: 0.1 mol/l NaHCO<sub>3</sub>, 2 mol/l urea, 0.1 mol/l sodium acetate buffer, pH 4.5, and 0.05 mol/l Tris-HCl buffer, pH 7.4. The sheets were then incubated overnight in the Tris-HCl buffer containing 0.5 % (w/v) bovine serum albumin. They were finally dried between filter papers and cut into strips measuring 5 x 70 mm. A mark was made 60 mm from the end of each strip.

Coupling of antibodies to Whatman chromatography paper No. 3 using periodate. Antibodies were also coupled covalently to chromatography paper oxidized by periodate according to Ferrua et al. (8). Four sheets, 70 x 150 mm, were washed 3 times in 500 ml distilled water. The sheets were then activated by incubation for 45 min in 0.1 mol/l sodium periodate. After activation they were quickly washed in distilled water followed by 0.1 mol/l carbonate-bicarbonate buffer, pH 9.0, before they were

incubated at room temperature for 18 h in 150 ml of the carbonate-bicarbonate buffer containing 150 mg rabbit IgG against human C-reactive protein. The sheets were washed consecutively in 0.1 mol/l  $\text{NaHCO}_3$ , 0.5 mol/l  $\text{NaHCO}_3$ , 0.1 mol/l sodium acetate buffer, pH 4.5, and 0.1 mol/l sodium phosphate buffer, pH 7.0, also containing 0.15 mol/l NaCl. This was followed by reduction for 1 h at 4°C with sodium borohydride (100 mg in 100 ml phosphate buffer, pH 7.0). The sheets were then washed with phosphate buffer and 0.05 mol/l Tris-HCl buffer, pH 7.4, and incubated overnight in the Tris-buffer containing 0.5 % (w/v) bovine serum albumin. The sheets were finally dried between filter papers and cut into strips measuring 5 x 70 mm. A mark was made 60 mm from the end of each strip.

Immunocapillarymigration. The capillarymigration of samples in the strips was performed as described in detail earlier (2; Fig. 1). Briefly, the unmarked end of a strip was inserted in a cup containing 300  $\mu\text{l}$  of sample. After the migration of the sample to the mark at 60 mm, the antigen-covered area was exposed in two steps. First, the strip was incubated for 3 min in a solution of horse-radish peroxidase-labelled antibodies. Excess peroxidase-labelled antibodies was washed off the strip by incubation for approximately 5 min in 0.05 mol/l Tris-HCl buffer, pH 7.4, containing 0.02 % (v/v) Tween 20. Second, the strip was incubated in a test-tube containing peroxidase substrate solution. A dense coloured area developed within 2½ min and the strip was removed from the solution. The height of the area was measured and compared with corresponding heights obtained from strips treated with a serial dilution of a standard and then developed as described above.

## RESULTS AND DISCUSSION

Filter paper and chromatography paper have been tried earlier as porous supports in immunocapillarymigration (1,2) but were found to bind fluorescein-labelled antibodies non-specifically to a high degree. The same problem was encountered when peroxidase-labelled antibodies were tried, but was overcome by an overnight incubation of the sheets in a solution of bovine albumin before they were dried and cut into strips.

Two methods for the covalent linkage of antibodies to paper were used here, periodate oxidation (8) and CNBr activation (9). The latter has been used earlier for coupling to cellulose acetate sheets (5) and was found to give reproducible coupling to chromatography paper when performed as described in this work. The method is disadvantageous since CNBr is toxic and irritant vapours are formed during the activation. The periodate technique, which has not got this drawback, was also tried. There were two critical steps when using this technique. First, the periodate solution used for oxidation caused a partial disintegration of the filter paper if the concentration of periodate was above 0.2 mol/l. A concentration of 0.06 - 0.1 mol/l gave a good and reproducible coupling yield without influencing the chromatographic properties. Second, the reduction with sodium borohydride had to be performed at 4°C. At room temperature the hydrolysis of borohydride is rapid and gas developed inside the paper destroys its structure. Therefore, the newly prepared sodium borohydride solution was chilled for 5 min at 4°C before reduction.

There were no differences between strips prepared by the two methods when they were used for quantitation of C-reactive protein in plasma samples. However, the periodate method was easier to use for large scale production of antibody containing sheets since periodate is non-toxic and no fume hood was required.

A survey of available filter and chromatography papers showed that Whatman chromatography paper No. 3 was best suited for use in immunocapillarymigration. This paper is rather thick, and is therefore easy to handle both in the dry and the wet state, and the flow rate is high. The migration rate for plasma or serum samples, diluted at least two-fold, was 60 mm per 5 min. Undiluted samples migrated slightly slower. As a result of the very rapid migration the total time required to complete an assay was only about 15 min (Fig. 1). In comparison with cellulose acetate strips (5) this is a decrease in time by 75 %. Since the reason for the use of a humidified chamber in our earlier experiments

(1,2) was the slow migration of samples in the porous supports used, the performance of the filter paper with and without the chamber was compared. Since no differences in migration rate or in the quantitative measurements could be detected, immunocapillarymigration in chromatography papers can be performed without the use of a humidified chamber.

By decreasing the amount of antibodies in the strips lower concentrations of antigen can be analyzed. Paper strips with decreasing amount of coupled antibodies against C-reactive protein were prepared by diluting the coupling solution with a solution of IgG from non-immune rabbit serum. The lowest concentration of C-reactive protein that could be clearly differentiated from zero was 0.3 mg/l. Thus the sensitivity is nearly the same as that obtained on cellulose acetate strips, 0.15 mg/l (5). A comparison of the standard curves obtained on paper strips with that obtained on cellulose acetate strips shows that the concentration range for which strips can be used is the same for the two materials (Fig. 2).

Duplicate determinations of the C-reactive protein concentration in ten plasma samples with the paper strips gave a standard deviation of 8 % of the mean, a precision comparable to that obtained with cellulose acetate strips (5).

In conclusion, the use of chromatography paper as the porous support in immunocapillarymigration has improved the technique further. Both normal (<5 mg/l) and elevated pathological concentrations of C-reactive protein in plasma samples can be determined within 15 min. Thus immunocapillarymigration is one of the most rapid methods for the determination of C-reactive protein.

#### ACKNOWLEDGEMENT

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## LEGENDS TO FIGURES

- Figure 1. A schematic drawing showing the immunocapillary-migration procedure as described in the text. The time in minutes required for each step when strips of chromatography paper were used is indicated to the right.
- Figure 2. Standard curves relating the heights of the C-reactive protein concentration of the sample solutions. Strips of Whatman chromatography paper No. 3 ■ and of cellulose acetate sheets ● .



Fig. 1.

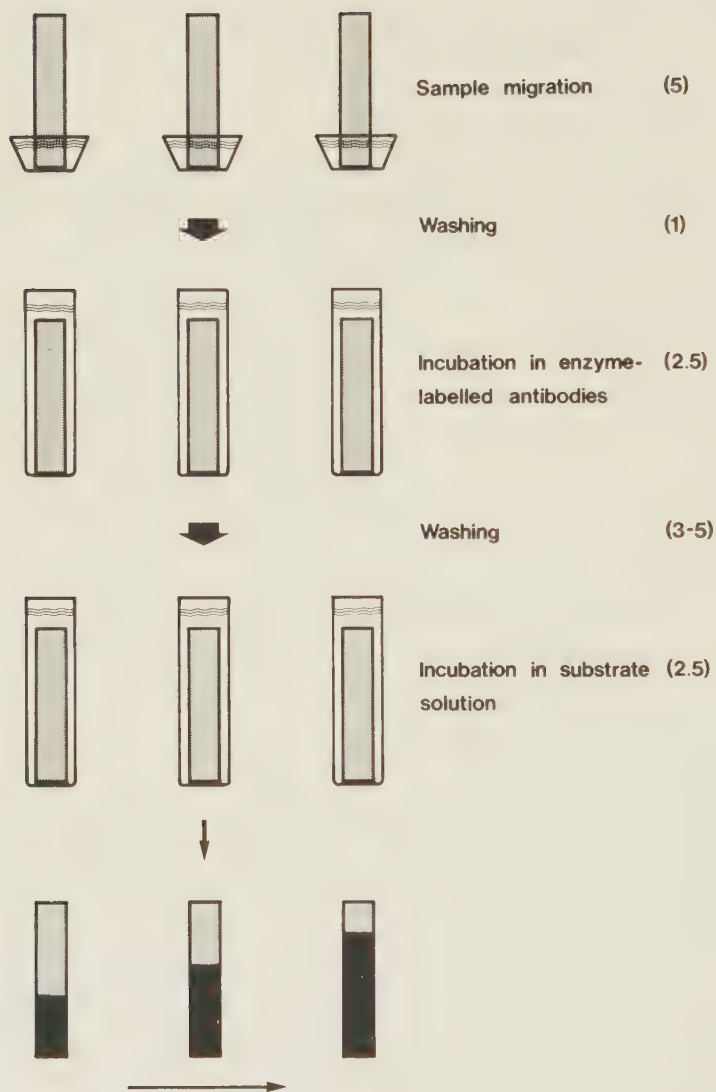
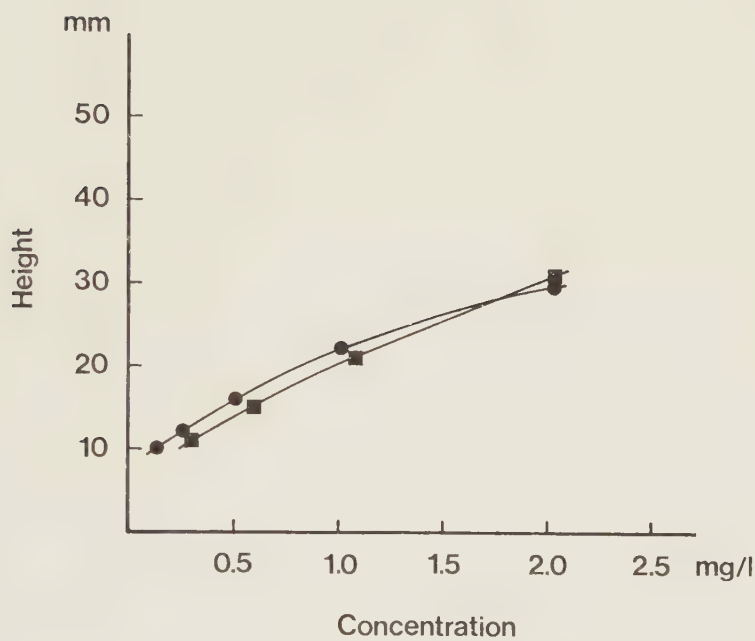


Fig. 2.









HIGH VOLTAGE ELECTROIMMUNOASSAY - AN ULTRARAPID METHOD FOR  
IMMUNOCHEMICAL QUANTITATION

Running titel: High voltage electroimmunoassay

by

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## ABSTRACT

The influence of the potential gradient-and of the buffer ionic strength on the time necessary to complete an electroimmunoassay was investigated. A decrease in the ionic strength and an increase in the potential gradient was found to decrease the time necessary for the completion of an electroimmunoassay. By the use of a combination of a high potential gradient and a low ionic strength it was possible to quantitate 10 of the major plasma proteins in time periods ranging from 9 min (for albumin) to 25 min (for IgG).



## INTRODUCTION

Quantitation of antigens by single radial immunodiffusion requires diffusion periods of up to 10 days (4). The introduction of electroimmunoassay as described by Laurell (2,3) meant that most antigens could be quantitated in time periods between 3 and 8 hours. Still more rapid quantitations can be performed by automated immune nephelometric procedures (6) but these require comparatively sophisticated and expensive equipment. In this work we investigate how the potential gradient and the buffer ionic strength influence on the time necessary to complete an electroimmunoassay. The work demonstrates that quantitations of antigens can be performed as quickly by electroimmunoassay as by automated nephelometric procedures provided that a suitable combination of low buffer ionic strength and high potential gradient is used.

## MATERIALS

Agarose was purchased from Marine Colloids Inc., Rockland, Maine, USA, Coomassie Brilliant Blue from Scharz/Mann, Orangeburg, N.Y., USA. All other chemicals were of reagent grade. Rabbit antisera against the investigated human plasma proteins were available at the laboratory.

## METHODS

Electroimmunoassay. The basic procedure of Laurell (3) was modified as described below.

The apparatus. The cooling surface of the apparatus described by Johansson (1) was made of glass instead of perspex to obtain better cooling efficiency. Circulating water of 4°C was used as cooling medium. The apparatus and the buffer reservoir were placed in a box of perspex and the electrodes were connected to the power supply via a circuit-breaker in the lid of the box to ensure maximal safety of the operator.

Buffer. In the buffer reservoirs and in the agarose bridges connecting the cooling surface of the electrophoretic apparatus with the buffer reservoirs 0.075 mol/l barbital buffer, pH 8.6, was used. In the antibody-containing agarose gel plate and in the agarose gel strips connecting the antibody-containing gel with the agarose bridges barbital buffers of pH 8.6 and varying between 0.010 and 0.075 mol/l were used.

Sample dilution and application. The samples were diluted with barbital buffer of the same composition as the one used for the preparation of the antibody-containing agarose gel. When high potential gradients (53 or 62 V/cm) were applied a solution of 0.1% agarose in the appropriate barbital buffer was used for the dilution of the samples.

Circular wells with a diameter of 2 mm and with their centres at least 6 mm apart were punched in the antibody-containing gel plate and 3  $\mu$ l of the diluted samples were applied.

Potential gradient. 8-62 V/cm was used.

## RESULTS

Electroimmunoassays of transferrin in several different dilutions of human plasma were performed with the use of potential gradients from 8 to 62 V/cm and with barbital buffers varying between 0.010 and 0.075 mol/l. For each combination of buffer concentration and potential gradient several electroimmunoassays with different electrophoretic times were performed. For each combination the time period necessary for the completion of the electroimmunoassay was considered to be the period after which a further extension of the electrophoresis time did not increase the length of any of the transferrin precipitates. Figure 1 gives the time period necessary for the completion of the electroimmunoassay of transferrin as functions of (A): the potential gradient and of (B): the buffer concentration, respectively. The height of the longest transferrin precipitate was about 35 mm for all combinations of potential gradient and buffer concentration. The figure demonstrates that the time period necessary for the completion of

an electroimmunoassay decreases with increasing potential gradient and with decreasing buffer concentration.

Figure 1 also shows that the cooling efficiency of the analysing system did not allow a combination of a high potential gradient with a high buffer concentration. When such combinations were tried the liquid in the sample wells and the gel between the wells dried very rapidly with a break of current as a result. When the samples were diluted with a 0.1 % agarose solution in the appropriate barbital buffer it was possible to use potential gradients up to 62 V/cm for the electroimmunoassay. When the samples were diluted with barbital buffers without agarose the highest potential gradient that could be used was 43 V/cm.

Table 1 gives the time periods necessary to complete electroimmunoassays of ten plasma proteins when a system with a potential gradient of 62 V/cm and with a 0.01 mol/l barbital buffer was used. As can be seen the time periods range from 9 to 25 min. As calculated from duplicate intra- or inter-plate determinations the coefficient of variation was found to be between 3 and 5 % for both high-range and low-range concentrations. Figure 2 gives an example of the precipitation patterns obtained with the use of a potential gradient of 62 V/cm.

In addition to allowing higher potential gradients to be used in the electroimmunoassays did the dilution of the samples with a 0.1 % agarose solution increase the distinctness of the precipitation lines obtained at lower potential gradients for several proteins. Figure 3 shows an example of this phenomenon.

## DISCUSSION

Since the electroimmunoassay was introduced (2) numerous modifications of the procedure have been described (7). But no systematic studies concerning the relation between the time necessary to complete an electroimmunoassay and the ionic strength and potential gradient used in the assay, have been published. A recent work by Renn and Evans demonstrated that the use of a buffer with low ionic strength in the antibody-containing gel significantly decreased the time necessary to complete an electro-

immunoassay (5). In this work we show that there are at least two ways to reduce the time necessary for electroimmunoassay.

The first way is to increase the potential gradient of the system. The limit of this way to decrease the time is set by the heat generated by high potential gradients. For when the cooling capacity of the system is exceeded the agarose gel will dry with an irregular electric field or even a disruption of the current as a result. The use of very high voltages is, in addition, a potential risk for the operator and requires therefore the use of safety arrangements.

When the samples were diluted with a 0.1 % agarose solution in barbital buffer it was possible to use higher potential gradients for the electroimmunoassays than when the samples were diluted with barbital buffer only. In addition, dilution of samples with agarose solution resulted in more distinct precipitation lines obtained at low potential gradients for several proteins. These beneficial effects of the use of the agarose solution as diluent are probably due to that this viscous solution establishes a comparatively homogeneous electric field and electroendosmotic flow around the application wells. An observation compatible with this interpretation is that the draining of the application wells during the electrophoretic run was much slower when the samples were diluted with the agarose solution than when they were diluted with plain buffer.

The second way to decrease the time of an electroimmunoassay is to decrease the ionic strength of the buffer used in the antibody-containing gel. Two requirements which the buffer in the antibody-containing gel must fulfil determine the limit of this way to decrease the time. The first requirement is that the buffer capacity must be high enough to maintain a constant gel pH during the electrophoretic run resisting e.g. the absorption of carbon dioxide from the air. The second requirement is that the ionic strength of the buffer must be high enough to keep antigens and antibodies of the system in solution until immunoprecipitation occurs. For it is well-known that some antigens e.g. IgM may precipitate spontaneously at low ionic strength. It is important to observe that the concentration of the buffer in the electrode res-

ervoirs can be, and preferably should be much higher than the concentration of the buffer in the antibody-containing gel.

The use of low ionic strength buffers in the antibody-containing gel in electroimmunoassays has several advantages. It allows rapid electroimmunoassays. It means that a weaker current flows in the antibody-containing gel and therefore that less energy and buffer chemicals are required and that in addition a less efficient cooling system can be used. The combination of a buffer of low concentration in the antibody-containing gel and a concentrated buffer in the electrode reservoirs means that a very large number of electroimmunoassays can be performed without change of the buffer in the electrode reservoirs.

This work demonstrates that by the use of a high potential gradient and low ionic strength buffer in the electroimmunoassay this procedure for immunochemical quantitation is very rapid. For example, it is possible to complete the immunoprecipitation of sixty samples applied to a gel plate containing antialbumin antiserum in 9 min. Since high voltage electroimmunoassay is at least as rapid as any described procedure for immunochemical quantitation and does not require any sophisticated equipment it seems to be a good alternative for immunochemical quantitation, especially when rapid results are desired.

#### ACKNOWLEDGEMENT

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TABLE I

Time for high voltage electroimmunoassay of ten human plasma proteins. A combination of 0.010 mol/l barbital buffer, pH 8.6 and 62 V/cm was used. The samples were diluted with a 0.1 % agarose solution in 0.010 mol/l barbital buffer, pH 8.6.

| Protein                   | Height of longest precipitate (mm) | Time for quantitation (min) |
|---------------------------|------------------------------------|-----------------------------|
| Albumin                   | 28                                 | 9                           |
| Antichymotrypsin          | 31                                 | 17                          |
| $\alpha_1$ -Antitrypsin   | 24                                 | 15                          |
| C-3                       | 20                                 | 10                          |
| IgA                       | 20                                 | 20                          |
| IgG                       | 27                                 | 25                          |
| $\alpha_2$ -Macroglobulin | 20                                 | 15                          |
| Prealbumin                | 27                                 | 25                          |
| RBP                       | 17                                 | 15                          |
| Transferrin               | 31                                 | 20                          |



## LEGENDS TO FIGURES

- Fig.1. Time for electroimmunoassay of transferrin in human plasma as a function of the potential gradient (A) with use of various buffer concentrations and as a function of the buffer concentration (B) with use of various potential gradients. Barbital buffer, pH 8.6 was used in all experiments.
- Fig.2. Precipitates obtained on high voltage electroimmunoassay of albumin in human plasma. A combination of 0.010 mol/l barbital buffer, pH 8.6 and 65 V/cm was used. The samples were diluted with a 0.1 % agarose solution in 0.010 mol/l barbital buffer, pH 8.6.
- Fig.3. Precipitates obtained on electroimmunoassay of ceruloplasmin in four dilutions of human plasma. A combination of 0.075 mol/l barbital buffer, pH 8.6 and 20 V/cm was used for the electroimmunoassay. The four samples to the left were diluted with barbital buffer only while the four samples to the right were diluted with a 0.1 % agarose solution in the barbital buffer.

Fig. 1A

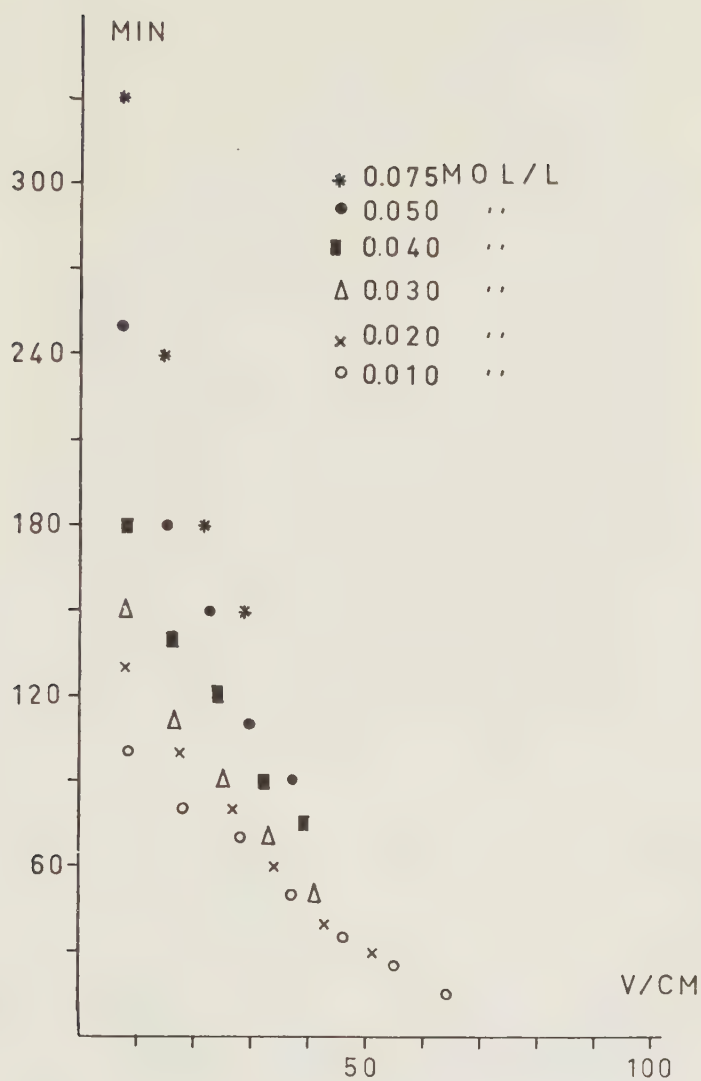


Fig. 1B

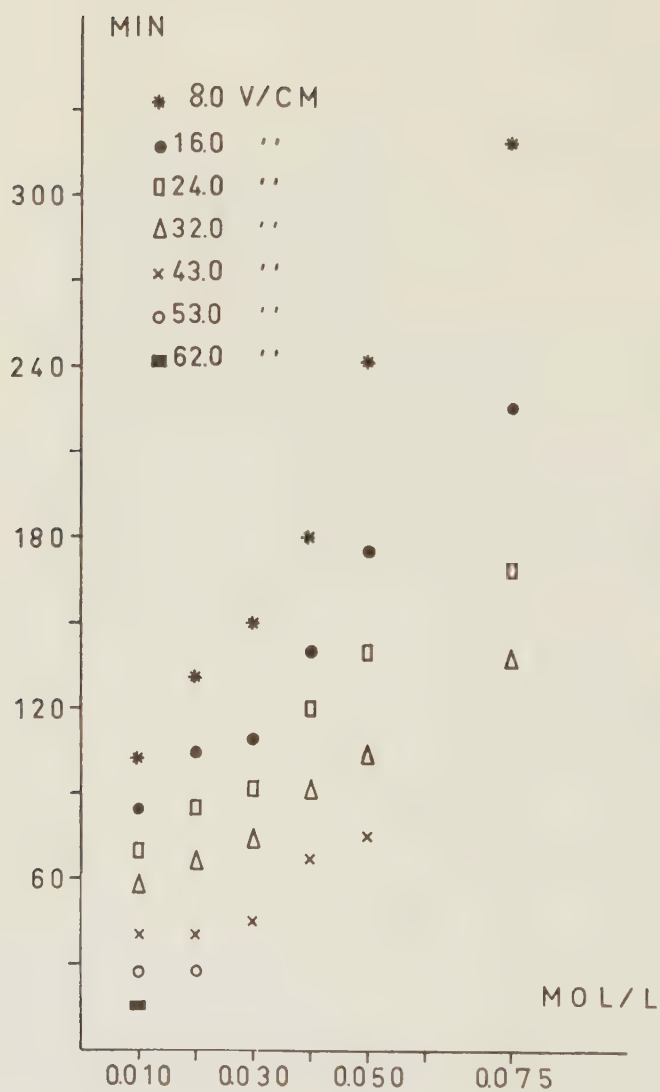
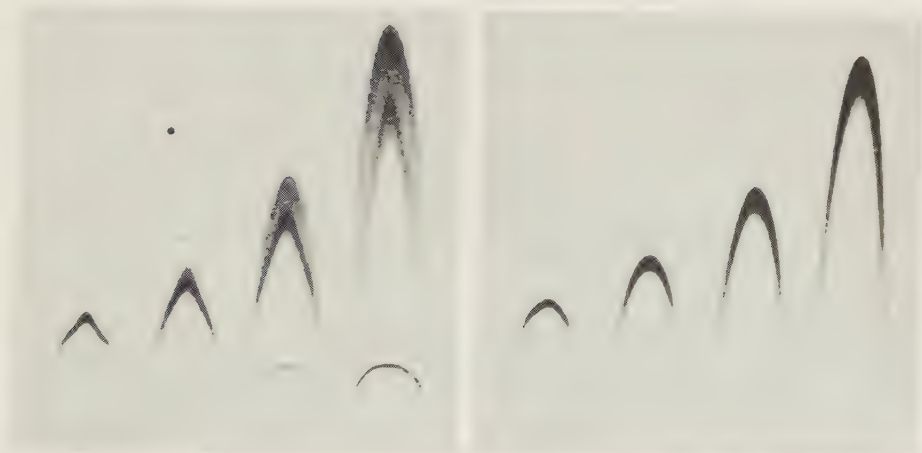


Fig. 2.



Fig. 3.











QUANTITATIVE CROSSED IMMUNOELECTROPHORESIS - A CONVENIENT  
PROCEDURE FOR THE SIMULTANEOUS QUANTITATION OF VARIOUS  
MOLECULAR FORMS OF AN ANTIGEN

Running title: Quantitative crossed immunoelectrophoresis

by

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## ABSTRACT

A procedure for high resolving quantitative crossed immuno-electrophoresis is described. It is a modification of Laurell's original procedure for crossed immunoelectrophoresis and includes the use of an internal standard of reduced and carboxymethylated ovalbumin. Its possible use for simultaneous quantitation of various molecular forms of an antigen is stressed.

## INTRODUCTION

Crossed immunoelectrophoresis as described by Laurell (5) and Garrot (3) has a high resolving power but gives only semiquantitative information. A modification of the procedure described by Clarke and Freeman (1,2) allows quantitative analysis but has a lower resolving power than the original technique (3). This work describes how the original technique can be modified so as to permit quantitative analysis without loss of resolving power.

## MATERIAL

Agarose was purchased from Marine Colloids Inc., Rockland, Maine, USA, Ultrogel AcA 54 from LKB-produkter, Bromma, Sweden, Coomassie Brilliant Blue from Schwarz/Mann, Orangeburg, N.Y., USA, complete Freund's adjuvant from Difco Laboratories, Detroit, Mich., USA and ovalbumin 99% electrophoretically pure, from Sigma, St. Louis, USA. All other chemicals were of reagent grade.

Monospecific rabbit antisera against human antichymotrypsin and transferrin were available at the laboratory. Fresh samples of human plasma were obtained from the routine laboratory.

## METHODS

Analytical and preparative agarose gel electrophoresis. The procedures described by Johansson were used (4).

Crossed immunoelectrophoresis. The system of Laurell was used (3,5).

Electroimmunoassay. The system described by Laurell was used (7).

Chromatography. Gel chromatography on Ultrogel AcA 54 was performed as described by the supplier.

Reduction and carboxymethylation of ovalbumin. 100 mg ovalbumin was dissolved in 10 ml 0.075 mol/l barbital buffer, pH 8.6, containing 2 mmol/l ethylenediaminetetra-acetic acid (barbital buffer). 400  $\mu$ l of barbital buffer containing 12.5 mg dithiothreitol was then added to the solution and after 2 hours at room

temperature, 800  $\mu$ l of a 2 mol/l tris-(hydroxymethyl)- amino-methane buffer, pH 8.6 containing 31.5 mg iodoacetic acid.

Purification of reduced and carboxymethylated ovalbumin. Analytical agarose gel electrophoresis revealed that the reduced and carboxymethylated ovalbumin preparation contained trace amounts of proteins with a slower electrophoretic migration than the bulk of the carboxymethylated ovalbumin. The protein was therefore further purified by preparative agarose gel electrophoresis followed by gel chromatography of pooled selected fractions on a column of Ultrogel AcA 54 in 0.05 mol/l ammonium bicarbonate. The final reduced and carboxymethylated ovalbumin preparation gave only one narrow band on analytical agarose gel electrophoresis and only one protein-containing peak on gel chromatography on Ultrogel AcA 54. The pure carboxymethylated protein was dissolved in barbital buffer at a concentration of 0.5 mg/ml. Small aliquots of this solution were stored at  $-20^{\circ}\text{C}$  until used.

Purification of antiserum against carboxymethylated ovalbumin.

1 mg reduced and carboxymethylated ovalbumin was dissolved in 1 ml 0.15 mol/l NaCl and the solution was emulsified with 1 ml complete Freund's adjuvant. The emulsion was then injected into the back feet and into several subcutaneous sites in two rabbits. The rabbits were given similar booster injections 2 weeks later and were then bled every second week. An antiserum-pool was established specificity tested by crossed immunoelectrophoresis with use of several concentrations of the antiserum and with varying amounts of human plasma or carboxymethylated ovalbumin in the first electrophoretic step. Human plasma gave no precipitation lines with the antiserum whereas carboxymethylated ovalbumin produced one strong precipitation line.

Quantitative crossed immunoelectrophoresis with reduced and carboxymethylated ovalbumin as internal standard. The procedure of Laurell (5) and Ganrot (3) is modified as follows.

Specified volumes of different dilutions of the antigen standard solutions or of the solutions to be quantitatively analysed are mixed with a small specified volume of a solution of reduced and carboxymethylated ovalbumin. The mixtures are then subjected to agarose gel electrophoresis as described by Johansson (4) and with rectangular slits measuring 0.3-0.8x10 mm. After the

electrophoresis a 3 mm wide gel strip parallel to the electrophoretic run and containing the separated antigens is cut out and placed in a trough of identical size in an agarose gel containing antisera against the antigens to be analysed and against reduced and carboxymethylated ovalbumin. An electric field is then applied perpendicular to the length axis of the gel strip until all antigens have precipitated. The agarose gel is then dried and stained. The gel with its supporting glass plate is then covered with a transparent or semi-transparent paper and illuminated from below. Drawings of the precipitation lines and of the upper edge of the inserted gel strip are then made and the areas outlined by the arcuate precipitates and the upper gel edge are measured by cutting out and weighing. For each standard dilution and sample, the ratio between the area outlined by the precipitate of the antigen to be quantitated and the area outlined by the ovalbumin precipitate is calculated. A standard curve relating the area-ratio to the antigen concentration is then constructed and used to calculate the concentration of antigen in the samples.

## RESULTS

The procedure described in this work was tested by applying it to the quantitation of transferrin and antichymotrypsin in human plasma. Figure 1 shows a run of serial dilutions of a standard solution containing human transferrin and antichymotrypsin. It demonstrates that while the area beneath the ovalbumin precipitate is fairly constant those beneath the transferrin and antichymotrypsin precipitation lines decrease with increasing dilution of the standard solution.

Figure 2A and B compare the concentration of transferrin and antichymotrypsin in 20 human plasma samples as determined by electroimmunoassay and by quantitative crossed immunoelectrophoresis. The correlation coefficients between the methods were 0.99 for antichymotrypsin and 0.98 for transferrin.

Calculated from duplicate determinations the standard deviation of quantitative crossed immunoelectrophoresis was found to be 4.3 per cent of the mean for antichymotrypsin and 7.7 per cent

of the mean for transferrin. Corresponding figures for the electroimmunoassay was 3.1 per cent of the mean for antichymotrypsin and 2.8 per cent for transferrin. When the areas beneath the ovalbumin precipitates were omitted from the calculations and only the areas under the antichymotrypsin and transferrin precipitates were used to construct the standard curves and to determine the antigen concentrations in the plasma samples the precision of the determinations decreased considerably. Coefficients of variation of 9.3 per cent and 9.9 per cent were obtained for transferrin and antichymotrypsin, respectively.

## DISCUSSION

Crossed immunoelectrophoresis as described by Laurell (5) and in more detail by Ganrot (3) has a high resolving power which for a greater part is due to the application of the antigen solutions in very narrow slits in the first electrophoretic step of the procedure. Although this allows an excellent separation of the antigens it also makes the method less convenient for quantitative determinations since it means that the amount of antigen transferred with the gel strip to the antibody-containing gel will vary considerably from run to run. One reason for this is that separate application slits will vary in shape due to e.g. varying ambient temperature and air humidity. Another reason is that it is difficult to apply a specified small volume of an antigen solution into a narrow slit. Still another reason is that the liquid inserted into the narrow slit will distribute unevenly and that the distribution probably will vary from slit to slit.

This work demonstrates that the original technique for crossed immunoelectrophoresis can be used for strictly quantitative work provided that an internal standard is used. The use of an internal standard will compensate for the transference of varying amounts of antigens associated with the use of narrow slits in the first electrophoretic step of the procedure. It is important to observe that this simple modification of the original technique does not decrease the resolving power of the procedure. Clarke and Freeman have earlier described a modification of crossed



immuno-electrophoresis which permits quantitative determinations (1,2). In their modification the zone electrophoretic step of the original procedure is significantly altered since the antigen solution is applied in a round hole 2 mm in-diameter instead of in a narrow slit and since a 15 mm wide agarose strip containing all of the applied antigens is transferred to the antibody-containing gel instead of a 3-5 mm wide gel strip containing only a part of the applied antigens. This alteration of the zone electrophoretic step decreases its resolving power to a certain degree and therefore the resolving power of the entire procedure. Another inconvenience with the modification of Clarke and Freeman is that it may be difficult to determine the base line for the correct calculation of the area beneath a precipitation peak (2). The reason is that the width of the antigen-containing gel strip allows a certain migration of antibodies into this strip before the immunoprecipitation lines are formed. Despite these limitations the technique of Clarke and Freeman has proven very useful for quantitative work.

In our modification of crossed immuno-electrophoresis reduced and carboxymethylated ovalbumin was used as internal standard for two reasons. Firstly, this avian antigen does not crossreact with any human antigen and probably not with any other mammalian antigen at least as long as mammalian antisera are used. Secondly, the reduction and carboxymethylation of the molecule excludes its interaction with other antigens via free sulphydryl groups.

Many antigens occur in several molecular forms in biological fluids (6,8,9). Although all of the molecular forms may react with a monospecific antiserum the stoichiometry of the reactions will usually differ. It is therefore difficult, not to say impossible, to make a reliable determination of the concentration in a biological fluid of such a heterogeneous antigenic population by means of ordinary methods for immunochemical quantitation like single radial immunodiffusion or electroimmunoassay. A correct immunochemical quantitation would require a separation of all molecular forms from each other and separate determinations of all forms of the antigen with use of separate standards containing the appropriate molecular form of the antigen. A separation of various mole-



cular forms of an antigen by commonly used procedures like gel and ion exchange chromatography is often troublesome and tedious.

Crossed immunoelectrophoresis can often be used to separate various molecular forms of an antigen from each other and is a simple and rapid technique. We therefore believe that the procedure for high resolving quantitative crossed immunoelectrophoresis described in this work will be a very useful aid in the quantitation of antigenic populations containing various molecular forms of the antigen.

#### ACKNOWLEDGEMENT

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## LEGENDS TO FIGURES

Fig.1. Crossed immunoelectrophoresis of six dilutions of a plasma standard pool containing transferrin and antichymotrypsin and, in addition, the internal standard reduced and carboxymethylated ovalbumin. The antibody-containing gel contained a mixture of rabbit antisera against the three antigens. The anodes in the first electrophoretic step in two of the runs are marked by plus signs. The three precipitation lines represent (from anode to cathode): reduced and carboxymethylated ovalbumin, antichymotrypsin and transferrin.

Fig.2. Relations between the quantitations of transferrin (A) and antichymotrypsin (B) in 20 human plasma samples by quantitative crossed immunoelectrophoresis (QCIE) and electroimmunoassay (EIA). The concentrations were expressed as percentages of a reference plasma pool. The equation  $y = 0.996 x + 0.4$  (for the transferrin estimations) and  $y = 0.993 x + 4.7$  (for the antichymotrypsin estimations), where  $y$  is the estimation by QCIE, give the mathematical relation between the estimations as calculated by the method of least squares.

Fig. 1.

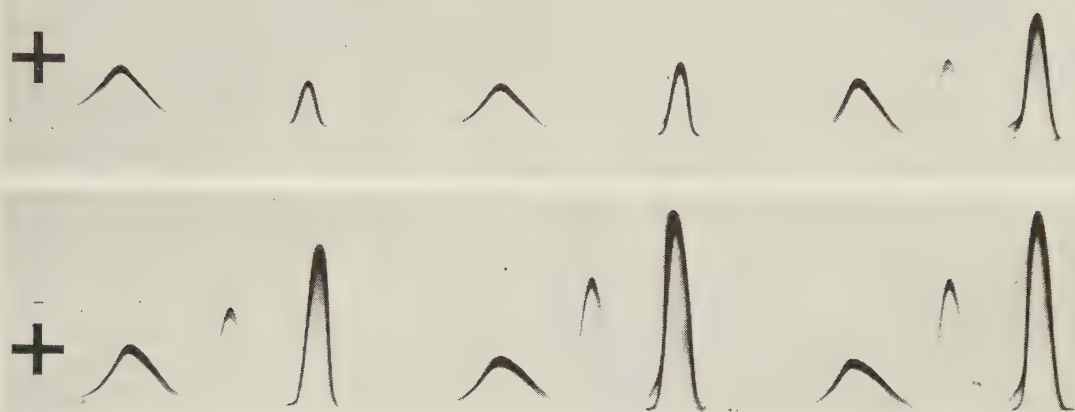


Fig. 2.

